厚生労働科学研究費補助金（医療機器開発推進研究事業）
平成 23 年度　分担研究報告書

ウイルス感染症への応用

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研究要旨

Respiratory Syncytial virus （RS ウイルス）がヘパリン固定化金ナノ粒子（Heparin immobilized Gold Nano Particle、Heparin-GNP）またはデキストラン硫酸固定化金ナノ粒子（DS25-GNP）によって濃縮できるか否かを調べた。ウイルス粒子とそれぞれの GNP を混合し、GNP を遠心沈殿させ、沈渣と上清中のウイルス量を定量 PCR 法で測定し比較した。その結果、RS ウイルスのサブタイプ A は DS25-GNP に結合するが、Heparin-GNP には結合しない可能性が得られた。サブタイプ B も Heparin-GNP には結合しなかったが、DS25-GNP への結合は不明であった。

A. 研究目的

糖鎖固定化金ナノ粒子を用いて RS ウイルスを濃縮すること。

B. 研究方法

各サブタイプRS ウイルスを含む液を 10,000g、15ー20 分、1 回遠心した上清を 0.45μm フィルター濾過したものにヘパリン固定化金ナノ粒子（Heparin Gold Nano Particle、Heparin-GNP）またはデキストラン硫酸固定化金ナノ粒子（DS25-GNP）を加え、30 分ー1 時間室温で反応させた。10,000g、30 分遠心し GNP を沈殿させ、その上清は 100,000g、60 分超遠心しウイルス粒子を沈殿させた。それぞれの沈殿から RNA を抽出し、cDNA を作り、SYBR グリーンを用いた Real-time PCR でウイルス量を定量した。

C. 研究結果

サブタイプ A の Ct 値は、Heparin-GNP 処理の上清の方がその沈殿よりも低かったが、DS25-GNP 処理では沈殿の方がその上清よりも低かった。サブタイプ B でも Heparin-GNP 処理の上清の方が Ct 値はその沈殿よりも低かったが、DS25-GNP 処理では一定の結果が得られなかった。

D. 考察

RS ウイルスのサブタイプ A は DS25-GNP に結合するが、Heparin-GNP には結合しない可能性が得られた。サブタイプ B も Heparin-GNP には結合しなかったが、DS25-GNP への結合は不明であった。

E. 結論

DS25-GNP で RS ウイルスを濃縮することができる可能性が得られた。この方法で患者鼻汁中のウイルスの検出感度を高めることができる可能性が示唆された。

F. 研究発表

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H. 知的財産権の出願・登録状況

なし

厚生労働省科学研究費補助金（医療機器開発推進研究事業）
平成23年度　分担研究報告書

各種神経・筋疾患におけるヘルペス感染の関与

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研究要旨

帯状疱疹を伴うVZV髄膜炎の症例の4例すべてで、髄液VZVが陽性になった。一部、唾液VZVが陽性になる症例があったが、2例とも口腔内（口腔粘膜）に帯状疱疹が認められた症例であった。唾液の検査がすべての髄液検査の代用になる訳ではなかったが、臨床的な十分に有用な検査となっている。

末梢性顔面神経麻痺の4例中2例に唾液でのVZV陽性が認められたが、口腔内（口腔粘膜）に帯状疱疹が認められた症例であり、体表の皮膚や粘膜に発疹のない症例でのヘルペス属PCR陽性は、今回の検討では認められなかった。今後、一層の症例の蓄積が求められる。

A.　研究目的

本研究事業では、PCRで検出できない極めて低濃度のウイルスを検出できる先端医療技術を行う計画であり、分担研究者らは特にヘルペス属ウイルス(Herpes simplex virus type 1 & 2(HSV1/2), Varicella zoster virus (VZV) , Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Human herpesvirus 6 (HHV-6))を対象として解析する。神経・筋疾患(髄膜炎/脳炎、顔面神経麻痺、帯状疱疹、神経痛、ウイルス性筋炎など)の中には、ヘルペス属ウイルスの関与が考えられており、これらの疾患の発症と早期発見を目的とした。髄液検査は、患者にとってその採取は比較的苦痛であるため、検査しやすい唾液などの検査がその代用にならないか？の検討も行った。

B.　研究方法

神経・筋疾患(髄膜炎/脳炎、顔面神経麻痺、帯状疱疹、神経痛、ウイルス性筋炎など)の中には、ヘルペス属ウイルスの関与が考えられているものがあり、これらの疾患を疑われ、診断された方に協力をお願いし、対象とした。

神経・筋疾患患者の髄液及び唾液、鼻腔ぬぐい液、喀痰などを採取。髄液は、医師が腰椎穿刺を行い、採取する。唾液や喀痰は滅菌スピッツに自分で排出することとした。検体は、1ー2日以内に鹿児島大学大学院理工学研究科の隅田研究室に搬送し、同研究室で遺伝子検査を行った。(HSV, VZVは全例。患者の臨床状況に応じて、CMV,　HHV6, EBVを選択した。)

（倫理面への配慮）

鹿児島市立病院の治験審査委員会にて、本研究は審査され、承認されている。検体採取においては、患者の同意をもって検査をおこなっている。髄液検査は、患者にとってその採取は比較的苦痛であるため、臨床的に髄液検査の適応があるもののみ行った。検査しやすい唾液などの検査がその代用にならないか同時に検査を行うことで、その検討も行った。

C.　研究結果

患者数は、17例(2011年7月から2012年3月までの9ヵ月)。検体数は、55検体で、その内訳は、髄液：21検体　唾液：34検体であった。髄液は、常に唾液とペアでの採取を基本とした。

＜症例の内訳＞

髄膜炎

無菌性髄膜炎 non-herpes　5例

VZV髄膜炎(すべて帯状疱疹合併)　4例

末梢性顔面神経麻痺　4例
三叉神経痛(HSV-IgM 抗体上昇)　1例
非ヘルペス疾患　5例
（舌根扁桃炎、てんかん重積状態、CIDP、脊髄梗塞、意識障害）

髄液での検討では、ヘルペス属 PCR が陽性になったのは、VZV が 4 例で、HSV1/2, CMV,EBV, HHV6 が陽性となった例はなかった。髄液 VZV が陽性になった 4 例は、全例帯状疱疹を合併した VZV 髄膜炎であった。

唾液での検討では、ヘルペス属 PCR が陽性になったのは、VZV が 2 例 HHV6 が 1 例で、HSV1/2, CMV,EBV が陽性となった例はなかった。末梢性顔面神経麻痺に帯状疱疹と VZV 髄膜炎が合併した 2 例で、唾液 VZV が陽性になった。無菌性髄膜炎と成人発症 Still 病の合併例において、唾液 HHV6 が陽性となった。

日常臨床で、ヘルペスに対する抗ウイルス剤を使用される末梢性顔面神経麻痺において、唾液での検査の有用性を検討したが、4 例中 2 例の陽性にとどまった。

D. 考察

髄液 VZV が陽性になった症例は、すべて帯状疱疹を合併した VZV 髄膜炎の症例で、ある程度ウイルス量が多いことが予想される症例であった。唾液でも VZV が陽性になった症例は、末梢性顔面神経麻痺に帯状疱疹と VZV 髄膜炎が合併した 2 例であったのだが、口腔内（口腔粘膜）に帯状疱疹が認められた症例であった。唾液の検査が髄液の代用になる訳ではなかった。

臨床上、最も問題になる HSV 関連の脳炎、髄膜炎症例がなかったため、今後の検討課題となった。唾液 HHV6 陽性が 1 例あったが、その病的な意義に関しては、様々な文献をあたっても一定の結論出ていないため、今後の検討課題となった。

E. 結論

帯状疱疹を伴う VZV 髄膜炎の症例で、髄液 VZV が陽性になった。一部、唾液 VZV が陽性になる症例があったが、2 例とも口腔内（口腔粘膜）に帯状疱疹が認められた症例であった。唾液の検査がすべての髄液検査の代用になる訳ではなかった。帯状疱疹を伴う髄膜炎例のすべてで、髄液 VZV が陽性になっていることは、臨床的には十分に有用な検査と言える。

末梢性顔面神経麻痺から見ると、2 例に唾液での VZV 陽性が認められたが、口腔内（口腔粘膜）に帯状疱疹が認められた症例であり、体表の皮膚や粘膜に発疹のない症例でのヘルペス属 PCR 陽性は、今回の検討では認められなかった。今後、一層の症例の蓄積が求められる。

F. 研究発表

なし

G. 知的財産権の出願・登録状況

特許取得・実用新案登録・その他

なし

Ⅲ. 研 究 成 果 の 刊 行 に 関 す る 一 覧 表

研究成果の刊行に関する一覧（平成23年度）

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Ⅳ. 研 究 成 果 の 刊 行 物 ・ 別 刷

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Regular Article

Heparin modulates the conformation and signaling of platelet integrin αIIbβ3

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ABSTRACT

Introduction: The glycosaminoglycan heparin has been shown to bind to platelet integrin αIIbβ3 and induce platelet activation and aggregation, although the relationship between binding and activation is unclear. We analyzed the interaction of heparin and αIIbβ3 in detail, to obtain a better understanding of the mechanism by which heparin acts on platelets.
Methods: We assessed conformational changes in αIIbβ3 by flow cytometry of platelets exposed to unfractionated heparin. In human platelets and K562 cells engineered to express αIIbβ3, we assayed the effect of heparin on key steps in integrin signaling: phosphorylation of the β3 chain cytoplasmic tail, and activation of src kinase. We measured the heparin binding affinity of purified αIIbβ3, and of recombinant fragments of αIIb and β3, by surface plasmon resonance.
Results and conclusions: Heparin binding results in conformational changes in αIIbβ3, similar to those observed upon ligand binding. Heparin binding alone is not sufficient to induce tyrosine phosphorylation of the integrin β3 cytoplasmic domain, but the presence of heparin increased both β3 phosphorylation and src kinase activation in response to ligand binding. Specific recombinant fragments derived from αIIb bound heparin, while recombinant β3 did not bind. This pattern of heparin binding, compared to the crystal structure of αIIbβ3, suggests that heparin-binding sites are located in clusters of basic amino acids in the headpiece and/or leg domains of αIIb. Binding of heparin to these clusters may stabilize the transition of αIIbβ3 to an open conformation with enhanced affinity for ligand, facilitating outside-in signaling and platelet activation.

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Introduction

The glycosaminoglycan heparin is a potent anticoagulant that is extensively used in the prevention and treatment of cardiovascular disorders. However, the efficacy of heparin is limited in part by its effect on platelets. Patients receiving intravenous heparin commonly experience an immediate, transient but mild non-immune-mediated thrombocytopenia, associated with biochemical evidence of platelet activation [1–5]. Heparin-mediated platelet activation should be distinguished from the much rarer syndrome of heparin-induced thrombocytopenia, caused by the formation of antibodies to platelet factor 4 (PF4) in a complex with heparin [1]. Both in vivo and in vitro, unfractionated heparin appears to be a stronger stimulant of platelet activation than lower molecular weight heparins [6–10]. In previous work, we have characterized the heparin oligosaccharide that binds to platelets, and shown that heparin binds to platelet integrin αIIbβ3 (platelet glycoprotein IIbIIIa) [11–15]. Thus, it seems likely that heparin binding to αIIbβ3 is directly related to its stimulatory effects. Understanding the interaction of heparin with platelets will help in the development of safer anticoagulants.

Integrin αIIbβ3 is the major platelet surface receptor for fibrinogen and other RGD-containing proteins. Like other integrins, αIIbβ3 is a bidirectional receptor that undergoes conformational changes and induces intracellular signaling upon ligand engagement (outside-in signaling), as well as upon cell activation by soluble such as thrombin or ADP (inside-out signaling). Both processes contribute to a signaling cascade that ultimately results in profound morphological and biochemical changes in the platelet [16,17]. One of the first steps in this cascade is tyrosine phosphorylation of the integrin β3 cytoplasmic tail, which alters its association with the platelet

Abbreviations: GlcNS6S-IdoA2S, glucosamine N-sulfate (6-O-sulfate)-iduronic acid (2-O-sulfate); mAb, monoclonal antibody; PBS, phosphate-buffered saline; PRP, platelet-rich plasma; pY, phosphotyrosine; SPR, surface plasmon resonance.

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cytoskeleton, leading to shape changes, and ultimately to aggregation and release of mediators contained in platelet α granules [18].

Recent evidence indicates that heparin augments the ligand-induced phosphorylation of platelet cytoplasmic proteins involved in multiple signal transduction pathways, including through integrin αIIbβ3 [19]. In the work presented here, we show that heparin directly amplifies signaling through integrin αIIbβ3. Our experiments show that heparin augments ligand-induced conformational changes in αIIbβ3, as well as increasing tyrosine phosphorylation of both the integrin β3 cytoplasmic domain and associated src kinase. In addition, we map the binding sites of heparin on the αIIbβ3 molecule. Our results indicate that heparin-induced platelet activation is caused by the binding of heparin to αIIbβ3, with induction or stabilization of a more activated conformation. We find that heparin binds predominantly to the αIIb subunit, and suggest some potential heparin-binding sites within the receptor.

Materials and methods

Detailed procedures and additional figures are included in the Supporting Information for this paper. For all experiments, we used a standard unfractionated porcine mucosal heparin (Celsus, Inc., M.W. ~15,000 Da, 179 units/mg).

Flow cytometry of integrin activation

All blood samples were collected from normal volunteers after obtaining informed consent following protocols approved by the Institutional Review Board. Whole blood was collected into Vacutainers containing 3.2% buffered sodium citrate (Becton-Dickinson) and immediately centrifuged to obtain platelet-rich plasma (PRP). PRP was incubated with PBS, heparin (Celsus, 5 μg/ml final concentration), EDTA (5 mM final), or eptifibatide (Millennium Pharmaceuticals/Schering, 300 μM final) and saturating concentrations of fluorescent antibodies or isotype controls. Samples were fixed and analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Both forward and side scatter detectors were set on logarithmic scales, and the analysis gate was set on the major population of platelets. The percentage of positive platelets and their mean fluorescent intensity were calculated from single-parameter histograms of a minimum of ten thousand events from the gated region.

Western blot analysis of protein tyrosine phosphorylation

For analysis of integrin β3 phosphorylation in human platelets, PRP was incubated with eptifibatide and/or heparin, washed, then lysed and immunoprecipitated with an anti-β3 monoclonal antibody (mAb) (PM6/13, Millipore). Samples were analyzed by Western blotting (Western Breeze kit, Invitrogen) using a polyclonal rabbit antibody directed against a peptide from human integrin β3 containing phosphotyrosine (pY)759 (Santa Cruz Biotechnology). After detection of phosphorylated β3, the membranes were stripped (ReBlot Plus Strong, Millipore) and total β3 was detected using a mouse mAb (clone 1, BD Transduction Laboratories). Scanned films were analyzed using ImageJ (http://rsb.info.nih.gov/ij/index.html), Microsoft Excel, and Prism5 (GraphPad) software.

KαIIbβ3 cells, derived from K562 cells transfected with plasmids expressing integrins αIIb and β3, were cultured as previously described [20]. To assay integrin β3 phosphorylation, cells were washed and spread on fibrinogen-coated Petri dishes. Nonadherent cells were removed, the adherent cells were lysed directly in sample buffer, and total protein analyzed by Western blotting. For detection of src activation, polyclonal anti-pY416 and monoclonal rabbit anti-src (Cell Signaling Technologies) were used as the primary antibodies.

Preparation, expression, and purification of αIIb and β3proteins and fragments

Intact human αIIbβ3 was purified from platelet concentrates as previously described [15]. Recombinant fragments of αIIb and the extracellular domain of β3 were generated by PCR cloning into His-fusion vectors and expressed in BL21 (DE3) bacteria (αIIb fragments) or S2 insect cells (β3extracellular domain). The αIIb fragments encompassed amino acids 1–269 (Region 1), 263–559 (Region 2), and 547–867 (Region 3) (Supplemental Figs. 1 and 2). The β3 extracellular domain included amino acids 28–716. For purification of αIIb fragments, inclusion bodies were solubilized and purified on a cobalt affinity column following manufacturer's protocols (Talon, Clontech). The β3 extracellular domain was isolated from supernatants of S2 cells by affinity chromatography on Talon resin.

Surface plasmon resonance detection of heparin binding

A gold-coated sensor chip (Nippon Laser and Electronics Lab) was coated with glucosamine N-sulfate (6-O-sulfate)-iduronic acid (2-O-sulfate) (GlcNS6S-IdoA2S) and placed in the sensing channel of a 2-channel SPR instrument (SPR-670, Moritex, Yokohama, Japan) [21,22]. A control chip coated with D-maltose was placed in the reference channel. Proteins in 0.1% TritonX-100 in PBS were injected simultaneously into both channels. Binding was detected, and binding curves and kinetic parameters calculated, using the manufacturer's software.

Analysis of heparin binding sites and αIIbβ3 conformation

Analyses were performed on the crystal structure of αIIbβ3 reported by Zhu, et al (PDB ID: 3FCS) [23] using the program Cn3D (version 4.1; http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml).

Results

Heparin induces conformational changes in platelet integrin αIIbβ3

We have previously shown that integrin αIIbβ3 is the major binding site for heparin on platelets [15]. To determine whether heparin interaction directly modulates integrin αIIbβ3 conformation, we used flow cytometry to measure the binding of a panel of antibodies to activation-dependent epitopes on human platelets. These epitopes are masked in the resting form of αIIbβ3, and are exposed by conformational activation of the receptor and high-affinity ligand binding. The antibodies LIBS-1 and −6 bind to ligand-associated epitopes on integrin β3, while PMI-1 and PAC-1 bind to αIIb [24–26]. PRP from five different normal volunteers was incubated with the respective antibodies in PBS alone, or in the presence of 5 μg/ml heparin. Fig. 1A shows the change in the percentage of platelets positive for each epitope, expressed as the ratio (% with heparin/% without heparin). Heparin increased the binding of all the activation-dependent antibodies, from an average of 1.4- (LIBS-1) to 1.8-fold (PAC-1). The increases in binding of both LIBS-6 and PAC-1 in the presence of heparin were statistically significant ($p<.05$, t-test). The presence of heparin alone did not induce the expression of the activation-dependent marker p-selectin (Fig. 1A), suggesting that heparin binding alone was not sufficient to induce platelet activation.

The effect of heparin on ligand-induced conformational changes was assessed in a second set of experiments. PRP was incubated with the integrin antagonist eptifibatide, and the binding of LIBS-1 and 6 was measured in the presence or absence of heparin. Although eptifibitide prevents αIIbβ3 signaling and platelet aggregation [18], biophysical measurements indicate that eptifibitide binding can induce partial activation of the receptor [27]. Because a large

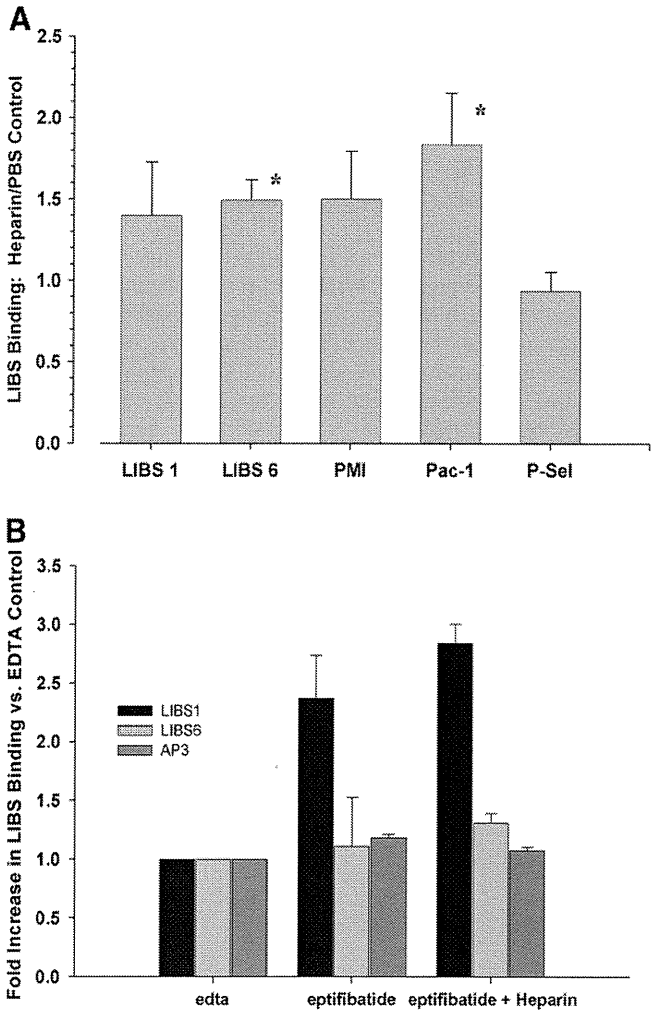

Fig. 1. Conformational changes in αIIbβ3 measured by flow cytometry. A, Relative binding of Pac-1, LIBS, and p-selectin antibodies to platelets in the absence of ligand. PRP was incubated with the indicated antibodies in the presence of 5 μg/ml heparin. Data are expressed as the percentage positive platelets for heparin treatment divided by PBS control (means ± SEM of 5 different subjects). The addition of heparin increased LIBS-1 and PAC-1 binding significantly more than that of p-selectin, which was no different than PBS controls ($p<.05$, *t*-test). B, Increased binding of LIBS1 but not LIBS6 in the presence of heparin and ligand. PRP was incubated with EDTA (5 mM) or eptifibatide (300 μM) with and without heparin (5 μg/ml). Separate aliquots were incubated with the conformation-dependent antibodies LIBS-1, LIBS-6, or AP3(which recognizes all conformations of αIIbβ3). The fold increase in mean fluorescent intensity of antibody binding over EDTA controls is shown. Values are the means ± SEM of 2 studies.

percentage of platelets were LIBS-positive in the presence of epitifibatide, further changes are expressed as the ratio of the mean fluorescence intensity in the presence or absence of heparin. Fig. 1B illustrates that the addition of heparin increases the binding of LIBS-1 in the presence of eptifibitide, while that of LIBS-6 is largely unaffected. Total surface expression of αIIbβ3, as measured by the antibody AP3, showed no changes with the different treatments.

Heparin augments signal transduction through integrin αIIbβ3

Ligand binding to integrin αIIbβ3 triggers signaling that ultimately results in platelet activation [16–18]. Because heparin appears to augment the ligand-bound conformation of αIIbβ3, we assayed the effect of heparin on tyrosine phosphorylation of the β3 cytoplasmic tail, one of the earliest signaling events of outside-in signaling. PRP was incubated with eptifibatide and varying amounts of heparin, and the phosphorylation of tyrosines in the β3 cytoplasmic tail was measured by Western blotting. To account for differences in gel loading, band intensities in the phosphotyrosine blots were normalized with the intensities of the total β3 integrin bands before assessing the effect of heparin addition. Fig. 2A shows that, while neither heparin nor eptifibatide alone stimulate tyrosine phosphorylation of the

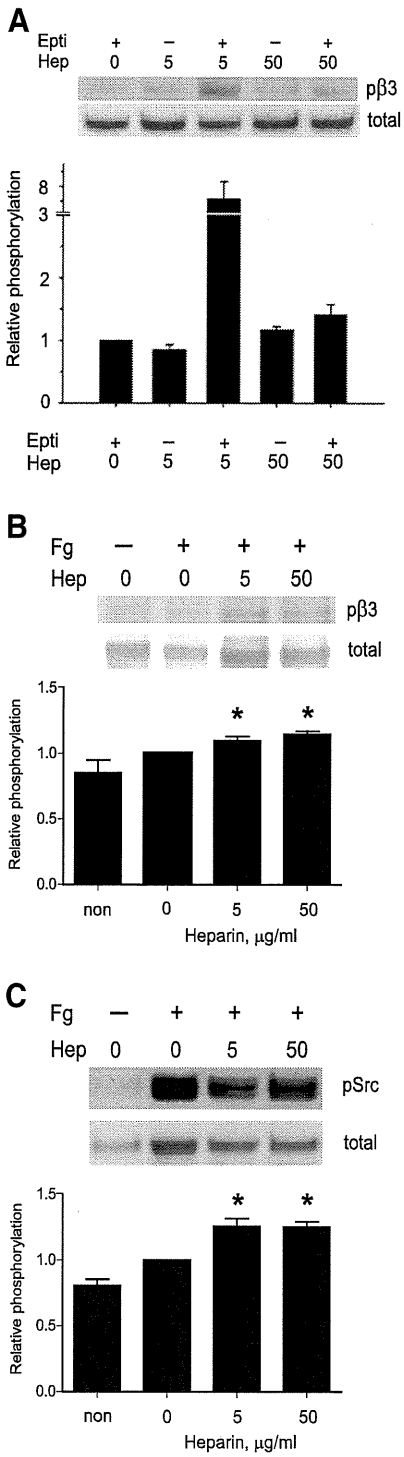

Fig. 2. Phosphorylation of the integrin β3 cytoplasmic tail and src in human platelets and KαIIbβ3 cells. A, Human PRP was incubated with eptifibatide (300μMolar) and/or heparin (5 or 50 μg/ml) and β3 phosphorylation measured as described in the Methods. Values are the means ± SEM of 2 independent experiments, expressed as the fold change in β3 phosphorylation compared to ligand alone. The Western blot of a representative experiment is depicted. B, Washed KαIIbβ3 cells were adhered to immobilized fibrinogen in the absence or presence of heparin (means ± SEM of 3 studies). Asterisks indicate $p<0.05$ (*t*-test) compared to the PBS control. C, Phosphorylation of src at tyr418 in KαIIbβ3 cells adhering to fibrinogen with or without heparin. Values are the means ± SEM of five experiments. Asterisks indicate $p<0.05$ (*t*-test) compared to control.

β3 cytoplasmic domain, a low concentration of heparin (5 μg/ml) significantly increased phosphorylation in response to eptifibatide.

To confirm that this effect was integrin-dependent, we repeated the experiment using K562 cells transfected with αIIbβ3 (KαIIbβ3) [20,28]. These cells have been shown to bind fibrinogen, and this model has been useful in dissecting integrin signaling. By flow cytometry, KαIIbβ3 cells do not express significant levels of other integrins (not shown). Fig. 2B illustrates that phosphorylation of integrin β3 in KαIIbβ3 cells adhering to immobilized fibrinogen is significantly increased in the presence of heparin. As in platelets, this effect is observed at low concentrations of heparin, and is not increased at higher concentrations.

Another early marker of outside-in signaling by integrin αIIbβ3 is activation of the tyrosine kinase src, which is constitutively associated with the β3 cytoplasmic tail [29]. In response to ligand binding, src is dephosphorylated at tyrosine-529 and phosphorylated at tyrosine-418, activating the kinase and initiating platelet activation. Fig. 2C shows that, in KαIIbβ3 cells adhering to fibrinogen, heparin significantly increases phosphorylation of endogenous src at tyrosine-418. These results indicate that heparin binding can amplify ligand-induced signaling through integrin αIIbβ3.

Identification of heparin-binding regions of αIIbβ3

The binding sites of heparin on the αIIbβ3 molecule were mapped more precisely by expressing components of the integrin heterodimer, and independently measuring their affinity for heparin structures, using surface plasmon resonance (SPR). To avoid complications arising from the structural heterogeneity of naturally occurring heparins, we immobilized a structurally defined disaccharide previously shown to bind to αIIbβ3 (GlcNS6S-Ido2S) on the SPR chip. Immobilization of the purified disaccharide also effectively increases the concentration

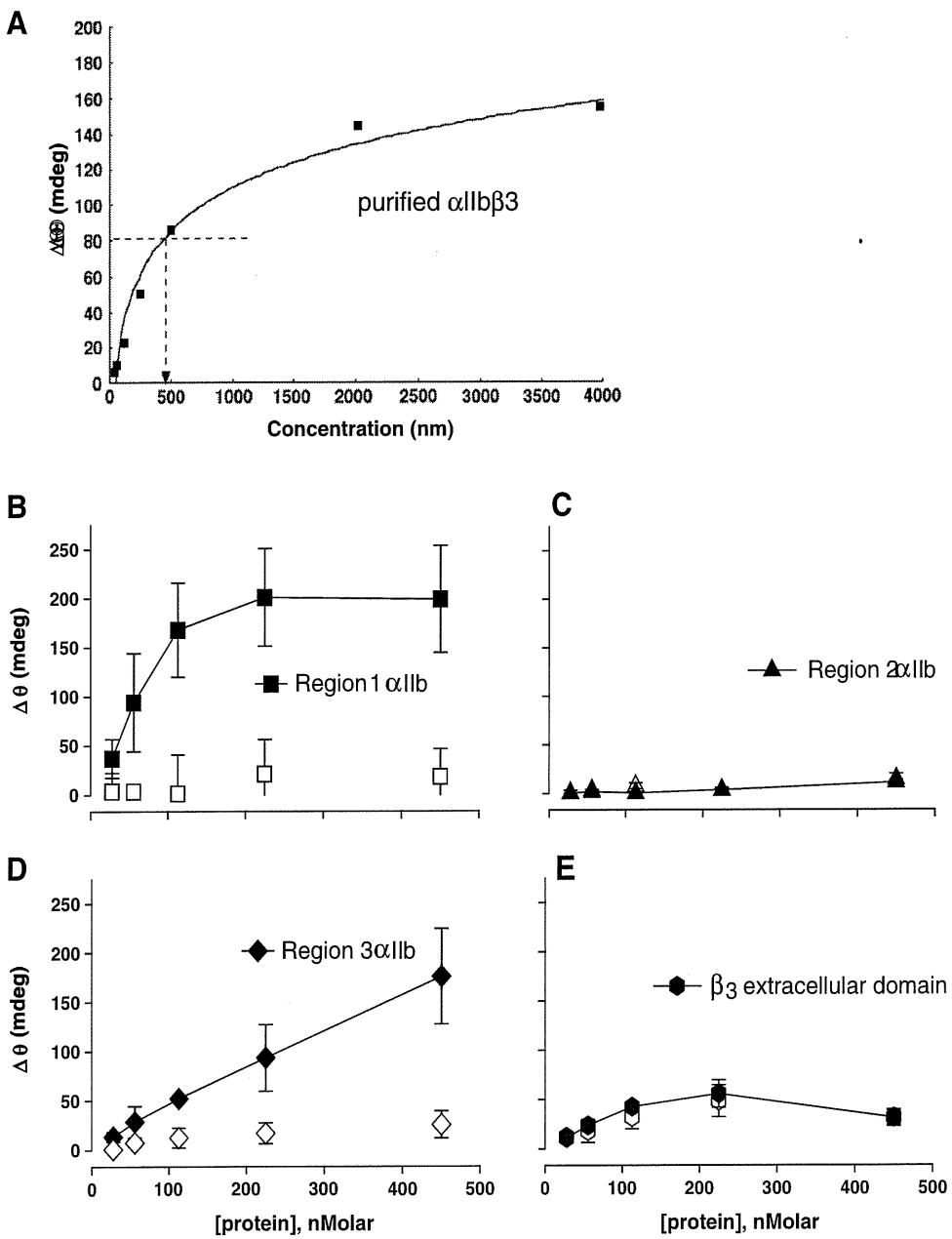

Fig. 3. SPR binding analyses. A, αIIbβ3. A typical binding experiment is illustrated, in which a range of concentrations of purified human αIIbβ3 were perfused over the sugar chip immobilized with a structurally defined synthetic oligosaccharide of GcNS6S-Ido2S known to bind to the platelet. B-E, recombinant fragments of αIIb and β3. For each fragment, binding to the defined heparin oligosaccharide is illustrated by the filled symbols, and binding to the control maltose chip by the open symbols. Values are the means ± SD from three independent experiments.

of the active structure [22]. Binding was first measured to intact integrin αIIbβ3 purified from human platelets, and then to isolated fragments of αIIb and β3 individually. Increasing concentrations of protein in buffer were passed over the immobilized oligosaccharide to assess binding. Fig. 3A illustrates the binding curve for purified platelet-derived αIIbβ3, where the K_D was calculated to be 440nM. Fig. 3 (B-E) are the binding curves for individual fragments of αIIb and the extracellular domain of β3, and Table 1 summarizes the dissociation constants derived from these experiments. Region 1 of αIIb (corresponding to the amino-terminal half of the head domain [30]) and Region 3 (containing part of the thigh and both calf domains) showed significant binding to the immobilized disaccharide. In contrast, the extracellular domain of β3 and αIIb Region 2 (the carboxy-terminal half of the head and adjoining thigh domain) did not appear to bind. These assays were repeated using unfractionated heparin immobilized on the SPR chip, which confirmed that only Regions 1 and 3 from αIIb bound to heparin (not shown), with affinities similar to the disaccharide. The calculated K_D for Region 1 was 68.1nM for the disaccharide (Table 1) and 106.6nM for unfractionated heparin.

Discussion

We have previously shown that heparin binds to platelet integrin αIIbβ3, resulting in platelet activation and aggregation [12,15]; however, in those experiments it was unclear if heparin binding alone could activate signaling. These experiments were undertaken to address this question. The flow cytometry experiments indicate that heparin binding can induce conformational changes in αIIbβ3 similar to those resulting from ligand binding, and that heparin augments the conformational changes induced by ligand binding. The absence of increased p-selectin expression in response to heparin alone suggests that platelets are not fully activated by heparin binding. Analyses of β3 tyrosine phosphorylation and src activation further confirm that heparin alone does not produce detectable outside-in signaling, but in the presence of ligand, heparin significantly enhances signaling through αIIbβ3, both in platelets and in transfected cells expressing the integrin. Finally, through analysis of heparin-binding to isolated fragments of αIIbβ3, we mapped putative heparin-binding sites to the headpiece and genu of αIIb.

Gao et al. have also recently reported that heparin potentiates signaling through platelet αIIbβ3 [19]. Their study demonstrated that heparin augments phosphorylation of FAK and Akt in response to ligand binding. This current report extends these findings by documenting that the earliest events of the outside-in signaling cascade are potentiated by heparin. By using the K562 model system, which is devoid of other platelet receptors, we were able to show that heparin binding increases phosphorylation of the integrin β3 cytoplasmic domain and activation of the associated src kinase in response to ligand binding. Taken together, these results indicate that heparin binding results in platelet stimulation at least in part by the induction or stabilization of an activated conformation of αIIbβ3, thus facilitating ligand binding to the integrin, and amplifying outside-in signaling and platelet activation.

Mapping the binding sites of heparin on the αIIbβ3 molecule suggests a model for this mechanism. Classical linear heparin-binding motifs composed of appropriately spaced cationic residues [31–33] can be found in the extracellular domain of β3 and Region 2 of αIIb. However, the results of the binding studies demonstrated that neither β3 nor Region 2 bound heparin, while Regions 1 and 3 of αIIb bound with significant affinity to heparin and its disaccharide. Additionally, the overall densities of arginine and lysine residues in Regions 1–3 do not correlate with their heparin-binding affinity (6.3% arginine/lysine residues in Region 1, 8.8% in Region 2, 5.7% in Region 3). Thus, it seems unlikely that heparin is binding to linear or random distributions of basic amino acids in the recombinant fragments, suggesting that the major heparin binding site(s) are formed by folding of the αIIb polypeptide. One caveat is that these recombinant proteins may not be folded in precisely the same conformation as the native protein.

Table 1
Dissociation Constants for purified αIIbβ3 and Recombinant Portions of αIIbβ3.

	K_D (nMolar)
native αIIbβ3	440
Region 1 αIIb	68.1
Region 2 αIIb	no binding
Region 3 αIIb	169
recombinant β3	no binding

The crystal structure of the αIIbβ3 extracellular domain [23] reveals that several clusters of arginine and lysine residues are located on the external face of the protein. Region 1 (which binds heparin) contains a large cluster on one face of the β-propeller (Fig. 4A and B). In particular, the arginines at positions 73, 77, 139, 140, and 208, along with lysine 118, are located around the cap subdomain of αIIb, adjacent to the residues essential for fibrinogen binding [34]. The basic amino acids contained in Region 3 (which also binds heparin) also appear to fall in several clusters (Fig. 4C and D). Two clusters are located on either side of the genu, in the thigh and calf-1 domains, and a third on the opposite face of calf-1.

This correlation between experimental binding studies and the crystallographic model of αIIb offers new hypotheses about how heparin modulates integrin function. Heparin may interact with the cap subdomain, thereby modifying the conformation of the ligand-binding site. In addition, binding of a charged polysaccharide chain to the clustered basic amino acids on either side of the genu could facilitate or stabilize the opening of the thigh and calf domains, extending the leg and exposing the ligand-binding site. Finally, the third cluster of basic amino acids on calf-1 appears to lie near binding sites for calcium in the β-propeller in the inactive conformation, so binding of heparin to this cluster could potentially modify interactions of αIIb with divalent cations [34]. Others have shown that opening of the leg alone can increase the affinity of αIIbβ3 for ligand. Mutation of residues surrounding the genu that constrain the opening of the leg inhibits activation-induced increases in binding affinity [35,36]. In contrast, introduction of a glycosylation site "behind" the genu, which prevents the leg from closing, results in constitutive high-affinity ligand binding [36]. Similarly, mutations of β3 that destabilize interactions with the αIIb leg, therefore favoring a more extended resting conformation, also confer constitutive high-affinity ligand binding [37]. Thus, heparin, by binding to one or more sites on the αIIb polypeptide, may stabilize an open, partially activated, conformation of αIIbβ3.

The demonstration that heparin can modulate the behavior of integrin αIIbβ3 may have implications beyond the regulation of platelet activation [14]. As an antithrombotic drug, unfractionated heparin may have theoretical drawbacks, particularly when administered to patients with systemic platelet activation, or in combination with integrin antagonists like eptifibatide that may synergistically stabilize activated conformations of αIIbβ3. Heparin has also been shown to affect processes such as tumor metastasis and leukocyte extravasation [38], in which the interactions of various integrins with their ligands appear to play a role in regulating cell adhesion. Thus, further understanding of the role of heparin in modulation of cell adhesion may advance the use of oligosaccharides derived from heparin for therapeutic purposes in cancer and inflammatory disorders, as well as in regulating coagulation.

Conflict of interest

No potential conflict of interest exists for any of the study authors.

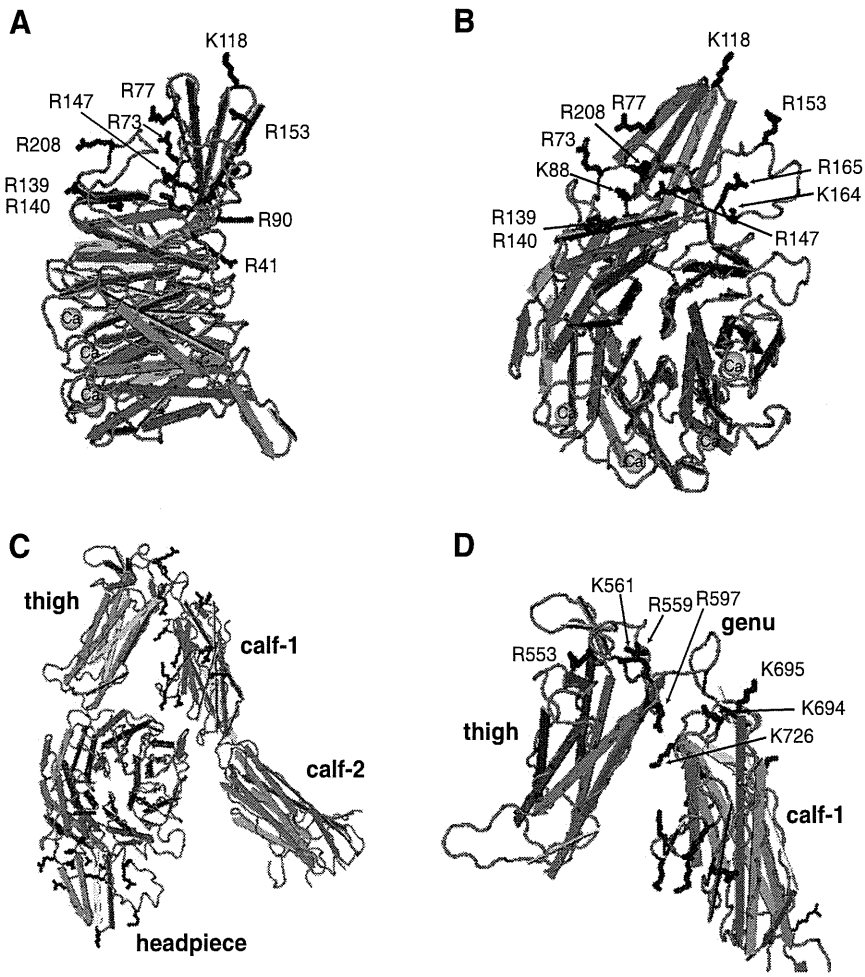

Fig. 4. Clustering of basic amino acids in αIIb. Locations of basic amino acids in αIIb, based on the crystal structure of Zhu, et al [26]. Figures were generated using Cn3D (version 4.1, http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). A & B, two views of the αIIb headpiece. B is rotated approximately 90° from A. The αIIb protein backbone is in green. The side chains of the Arg (R) and Lys (K) residues located in Region 1 (AA 1–262) are represented by blue sticks. C, an overview of the closed form of the αIIb chain. The Arg and Lys side chains in Regions 1 and 3 are depicted as blue sticks. **D**, the thigh and calf-1 domains of αIIb. The Arg and Lys residues within 15 Å of the genu are identified.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.thromres.2011.11.054.

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O-sulfate groups of heparin are critical for inhibition of ecotropic murine leukemia virus infection by heparin

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ABSTRACT

There is increasing evidence that soluble glycosaminoglycans such as heparin can interfere with the infectivity of various viruses, including ecotropic murine leukemia viruses (MLVs). The ecotropic MLV, Friend MLV (F-MLV) and the neuropathogenic variants A8 MLV and PVC-211 MLV, were susceptible to heparin-mediated inhibition of infection of NIH 3T3 cells. To investigate the interaction between the envelope glycoprotein (Env) of MLV and heparin, we prepared vesicular stomatitis virus-based pseudotyped viruses carrying the Env of F-, A8, or PVC-211 MLVs. Surface plasmon resonance analyses indicated that the Env of A8 and PVC-211 MLVs had a higher binding activity to heparin than that of F-MLV. We examined the influence of *N*- or *O*-sulfation of heparin on binding activity to Env and on the inhibition of the infectivity of MLV and pseudotyped viruses carrying Env. This analysis indicated that the *O*-sulfate groups of heparin play a major role in determining Env-dependent inhibitory effects.

Introduction

Previous studies have shown that soluble glycosaminoglycans (GAGs), such as heparin, inhibit the infectivity of ecotropic murine leukemia virus (MLV) (Batra et al., 1997; Guibinga et al., 2002; Jinno-Oue et al., 2001; Le Doux et al., 1996, 1999; Masuda et al., 1997; Walker et al., 2002). Jinno-Oue et al. (2001) found that heparin influences Env-dependent attachment of the virus to the cell surface. By contrast, Walker et al. (2002) and Guibinga et al. (2002) reported that heparin inhibits Env-independent interaction of MLV with target molecules on the cell surface. Infection by ecotropic MLV is mediated by the binding of the viral Env protein to the rodent ortholog of cationic amino acid transporter 1 (CAT-1), which serves as the specific cellular receptor (Albritton et al., 1989; Kim et al., 1991; Wang et al., 1991). Although the Env-receptor interaction appears to be required for membrane fusion and entry of the viral capsid, it has been reported that initial attachment of a retroviral particle to the cell surface can take place in a receptor-independent manner (Guibinga et al., 2002; Pizzato et al., 1999; Walker et al., 2002). The fact that soluble GAGs, such as heparin, inhibit MLV infectivity suggests that cell surface GAGs, such as heparan sulfate, might be involved in the initial attachment (Batra et al., 1997; Guibinga et al., 2002; Jinno-Oue et al., 2001; Le Doux et al., 1996, 1999; Masuda et al., 1997; Walker et al., 2002). Similarly, it has been shown that infection by other enveloped viruses, such as herpes viruses (Neyts et al., 1992; Secchiero et al., 1997; WuDunn and Spear, 1989), respiratory syncytial virus (Krusat and Streckert, 1997) and human immunodeficiency virus (HIV) (Mondor et al., 1998; Patel et al., 1993), are inhibited by heparin, and that cell surface attachment of these viruses involves cell surface GAGs. Thus, the interaction of viral particles with soluble and cell surface GAGs is an important issue for virology in general.

In the present study, we initially investigated the influence of heparin on infection of ecotropic MLVs including Friend MLV (F-MLV) clone 57 (Oliff et al., 1980) and its neuropathogenic variants, A8 MLV (Takase-Yoden and Watanabe, 1997; Watanabe and Takase-Yoden, 1995) and PVC-211 MLV (Kai and Furuta, 1984; Masuda et al., 1992). The infection of neonatal rats with A8 or PVC-211 MLV induces spongiform neurodegeneration without inflammatory infiltrates. The primary determinant for neuropathogenicity of these viruses has been identified as Env. Comparison of the amino acid sequences of A8-Env and PVC-211-Env showed that only 3 of the 676 amino acids differ. F-MLV is non-neuropathogenic and F-Env shows differences at 26 of the 676 amino acids compared to A8-Env. Here, we performed surface plasmon resonance (SPR) analyses to compare the binding activities to heparin of pseudotyped viral particles carrying Env of ecotropic MLVs. To our knowledge, this is the first time that SPR technology has been applied in a quantitative analysis of

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the interaction between viral particles carrying MLV Env and heparin. Heparin is a linear polysaccharide composed of α1-4 linked disaccharide repeating units. The most common unit contains 2-*O*-sulfated iduronic acid and 6-*O*-sulfated, *N*-sulfated glucosamine. The negatively-charged sulfate groups of heparan sulfate (which is structurally related to heparin) are thought to play an important role in its biological activity, such as fibroblast growth factor (FGF) signaling, and to act as an entry receptor for herpes simplex virus type 1 (Capila and Linhardt, 2002; Copeland et al., 2008; Shukla et al., 1999; Xia et al., 2002; Ye et al., 2001). We also examined the relative importance of *N*- or *O*-sulfation of heparin for its inhibitory effects on infection by ecotropic MLVs and on the binding activities to viral particles using chemically modified heparins: *N*-acetylheparin (NA-H), which has diminished *N*-sulfation; de-*N*-sulfated heparin (dNS-H), which completely lacks *N*-sulfation; and *N*-acetyl-de-*O*-sulfated heparin (NAdOS-H), which has markedly diminished *N*-sulfation and completely lacks *O*-sulfation. Taken together, the present investigations have characterized the inhibitory activities of heparin against ecotropic MLV infection of NIH 3T3 cells, while the SPR analysis showed that the binding activity of ecotropic MLV with heparin may be determined by Env amino acid sequences. We also demonstrate that the *O*-sulfate groups of heparin play a major role in inhibiting the infectivity of ecotropic MLV on cells in an Env-dependent manner. The possible mechanisms of the inhibition of heparin against viral infection and the implications of heparan sulfate on the cell surface are discussed.

Results

Inhibition of ecotropic MLV infection by heparin and its derivatives

We compared the effects of heparin and its derivatives on the infectivity of F-, A8, and PVC-211 MLVs using viruses that had been pre-incubated with various concentrations of heparin, or one of the derivatives, in the absence of polybrene. After this pre-incubation step, the virus-heparin or virus-derivative mixture was inoculated onto NIH 3T3 cells at a multiplicity of infection (MOI) of 1 in the presence of 10 μg/ml polybrene. We used polybrene for viral infection, because MLV infections are usually carried out in the presence of polybrene to enhance infection. After 72 h, viral production was evaluated by measuring virion-associated reverse transcriptase (RT) activities in the culture supernatants.

Pre-incubation of the viruses with heparin at concentrations greater than 1 μg/ml resulted in a dose-dependent decrease in viral production (Fig. 1A). The 50% inhibitory dose (ID_{50}) values of heparin for F-, A8, and PVC-211 MLVs were 12.7 ± 4.5, 8.5 ± 0.9, and 13.0 ± 3.3 μg/ml, respectively; these values were not significantly different (Table 1). The structure of the most common disaccharide unit of heparin is composed of 2-*O*-sulfated iduronic acid and 6-*O*-sulfated, *N*-sulfated glucosamine (Fig. 1E). To investigate whether differences in the sulfation patterns of heparin affect the ability to inhibit ecotropic MLV infection, we pre-incubated viruses with a heparin derivative: NAdOS-H, which has markedly diminished *N*-sulfation and no *O*-sulfation; NA-H, which has diminished *N*-sulfation; or dNS-H, which completely lacks *N*-sulfation. NAdOS-H did not show inhibitory effects on infection of NIH 3T3 cells with F-, A8, or PVC-211 MLV even at a concentration of 1000 μg/ml (Fig. 1B and Table 1). In contrast, NA-H inhibited production of F-, A8, and PVC-211 MLVs in infected NIH 3T3 cells, with ID_{50} values of 54.5 ± 4.1, 62.1 ± 2.5, and 53.7 ± 2.8 μg/ml, respectively (Fig. 1C and Table 1); these values were significantly higher than for heparin ($P<0.001$). dNS-H also inhibited infection of NIH 3T3 cells with F-, A8, and PVC-211 MLVs, with ID_{50} values of 76.8 ± 3.9, 65.4 ± 2.3, and 72.4 ± 4.2 μg/ml, respectively (Fig. 1D and Table 1). The ID_{50} values for dNS-H were significantly higher than those of heparin ($P<0.001$).

Preparation of vesicular stomatitis virus (VSV)-based pseudotyped viruses carrying ecotropic MLV Env

Previous studies suggested that heparin affects ecotropic MLV infection during the early steps of viral replication. We used pseudotyped viruses bearing Env to analyze the effects of heparin on the viral replication process from the attachment to gene expression steps. We also sought to clarify the contribution of Env of ecotropic MLV to the inhibitory effects of heparin against viral infectivity by examining this response in the absence of other retroviral proteins, such as Gag and Pol, that might influence heparin-mediated reduction in infectivity. To this end, we prepared VSV based-pseudotyped viruses carrying the Env of F-, A8, or PVC-211 MLV (VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, respectively) using 293T cells.

Initially, we performed a Western blot analysis using an anti-Env antibody to examine whether Env protein is normally expressed in the 293T cells transfected with F-, A8-, and PVC-211-Env expression vectors, and compared the expression levels of Env in these cells. In the transfected 293T cells, the primary product observed was gp70, although gpr85 was also detectable (lanes 1–3 in Fig. 2). The expression levels of Env protein were similar in cells transfected with F-, A8-, and PVC-211-Env expression vectors. The Env-expressing cells were infected with recombinant VSV, which has the green fluorescence protein (GFP) gene in place of the viral G protein gene, and VSV-pseudotyped viruses carring Env were obtained. As a control, VSV viruses-like particles lacking Env (VSV/ΔEnv) were also obtained as described in the Materials and methods section. Next, we compared the amount of Env protein packaged in VSV-pseudotyped viruses among VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env. In total, 2×10^5 infectious units of VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env were spun down and the precipitates were used in a Western blot analysis. With regard to viral particles, gp70 was observed, and the amount of Env protein showed no appreciable differences among the virions (lanes 5–7 in Fig. 2).

Inhibitory effects of heparin and its derivatives on the infectivity of VSV-based pseudotyped viruses carrying ecotropic MLV Env

We examined the inhibitory effects of heparin and its derivatives on the infectivity of VSV-based pseudotyped viruses carrying ecotropic MLV Env. VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env were pre-incubated with various concentrations of heparin, or a derivative, in the absence of polybrene, and then inoculated onto NIH 3T3 cells at an MOI of 1 in the presence of 10 μg/ml polybrene. After 16 h, GFP-positive cells were counted by fluorescence-activated cell sorting (FACS) in order to evaluate viral infectivity. Heparin was found to inhibit infection of NIH 3T3 cells with VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env (Fig. 3A) with ID_{50} values of 7.0 ± 0.3, 8.0 ± 0.3, and 7.4 ± 0.4 μg/ml, respectively (Table 2). Although NAdOS-H did not inhibit infectivity of VSV/F-Env, VSV/A8-Env, or VSV/PVC-211-Env on NIH 3T3 cells (Fig. 3B and Table 2), high concentrations of NA-H or dNS-H did show inhibition of infection of NIH 3T3 cells (Figs. 3C and D). The ID_{50} values of NA-H (50.1 ± 3.9, 53.4 ± 2.6, and 50.8 ± 3.7 μg/ml for VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, respectively) and of dNS-H (67.8 ± 1.5, 64.8 ± 2.0, and 71.5 ± 1.8 μg/ml for VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, respectively) were significantly higher than those of heparin ($P<0.001$) (Table 2).

We also sought to compare the effects of heparin and its derivatives on the infectivity of ΔEnv-VSV-pseudotyped viruses. However, the efficiency of GFP gene transduction of the cells with the ΔEnv-VSV-pseudotyped viruses was too low to allow a reliable comparison to be carried out.

SPR analysis of the direct interactions of ecotropic MLV Env with heparin or its derivatives

In order to analyze direct interactions between Env and heparin, we measured the binding activities of VSV/F-Env, VSV/A8-Env, and

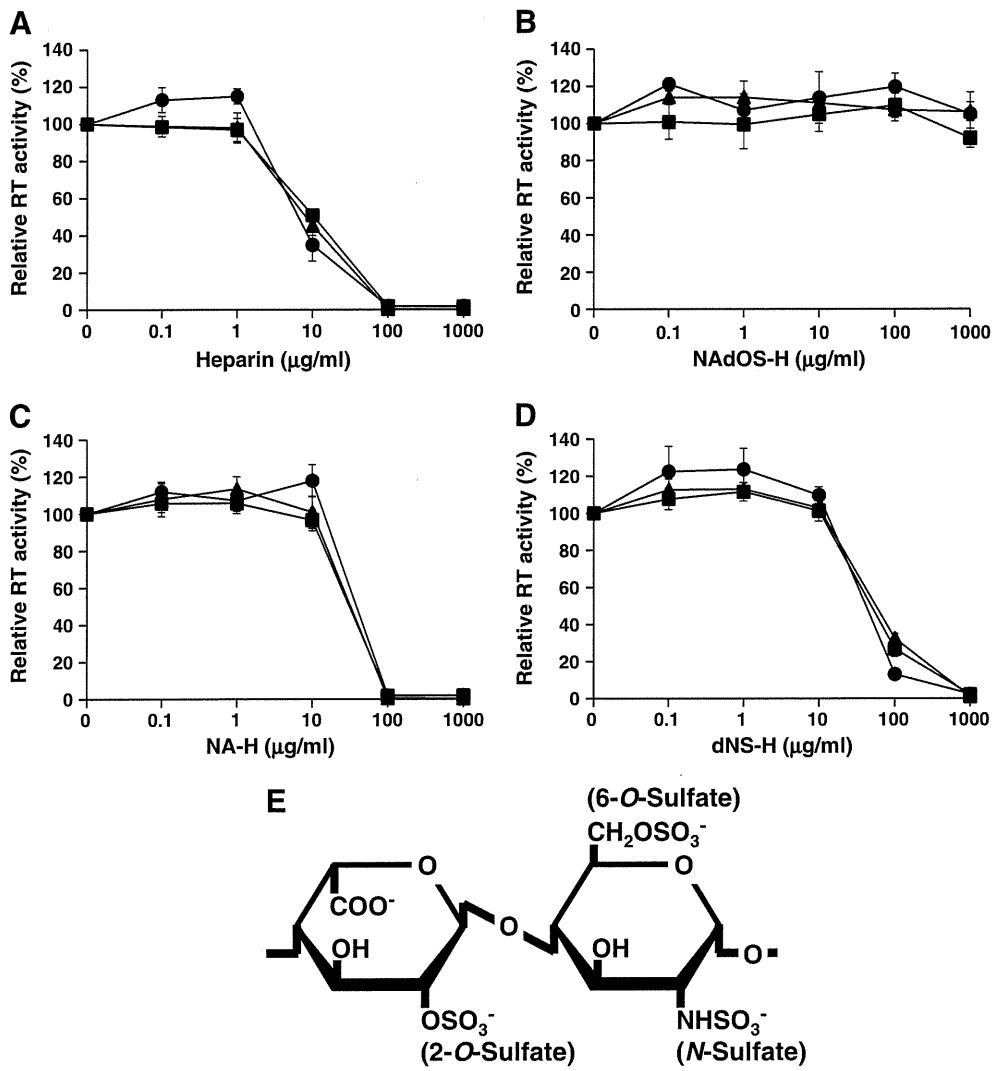

Fig. 1. Inhibitory activities of heparin and three derivatives against production of retroviruses in NIH 3T3 cells. F- (triangle), A8 (circle), and PVC-211 (square) MLVs were pre-incubated with various concentrations of heparin (A), NAdOS-H (*N*-acetyl-de-*O*-sulfated heparin) (B), NA-H (*N*-acetylheparin) (C), or dNS-H (de-*N*-sulfated heparin) (D) for 1 h at 37 °C in the absence of polybrene. The virus–heparin mixture was then inoculated onto NIH 3T3 cells in the presence of 10 μg/ml polybrene. After incubation for 1 h at 37 °C, the cells were washed three times with FCS-free DMEM and fresh culture medium was added. After 72 h, virion-associated RT activity in the culture supernatants was measured by an RT assay as described in the Materials and methods section. The mean values of 4 independent experiments and SEM are shown. (E) Schematic diagram of the major disaccharide repeating units of heparin. The most common disaccharide unit of heparin is composed of 2-*O*-sulfated iduronic acid and 6-*O*-sulfated, *N*-sulfated glucosamine.

VSV/PVC-211-Env to a heparin-immobilized chip (Heparin chip) by SPR. The SPR analyses were performed in the absence of polybrene; this protocol was adopted because in the experiments on inhibition of viral infection by heparin (Figs. 1 and 3), the viruses were pre-incubated with heparin in the absence of polybrene. Two different preparations of each pseudotyped virus were used for the analysis.

Table 1
ID_{50} of heparin and three derivatives against infection of MLVs into NIH 3T3 cells.

Reagents	Viruses		
	F	A8	PVC-211
Heparin	12.7 ± 4.5[a]	8.5 ± 0.9[a]	13.0 ± 3.3[a]
NAdOS-H	>1000	>1000	>1000
NA-H	54.5 ± 4.1	62.1 ± 2.5	53.7 ± 2.8
dNS-H	76.8 ± 3.9	65.4 ± 2.3	72.4 ± 4.2

ID_{50} values (μg/ml) were calculated from the data shown in Fig. 1. The mean values (and SEMs) of 4 independent experiments are shown. Statistical comparison was performed using the t test. NAdOS-H: *N*-acetyl-de-*O*-sulfated heparin; NA-H:*N*-acetylheparin; dNS-H: de-*N*-sulfated heparin.

[a] $P<0.001$ vs NA-H and dNS-H in each virus.

Env-deficient VSV-like particles (VSV/ΔEnv) and culture supernatant from 293T cells (Mock) were used as negative controls. Typical sensorgrams of the binding of the samples to the Heparin chip are shown in the top column of Fig. 4A. The flow of the viral particles, at various concentrations, across the Heparin chip was started at 0 s, and flow of the washing buffer was started at 300 s. Since the binding of virus to the Heparin chip appeared to reach equilibrium at 450 s, the Δdeg value at 450 s was selected as reflecting binding activity and plotted as shown in the top column of Fig. 4B. The results indicated that VSV-based pseudotyped viruses were bound to the Heparin chip in a dose-dependent manner. The binding activities of VSV/A8-Env and VSV/PVC-211-Env were clearly higher than that of VSV/F-Env, which exhibited higher binding activity than the negative controls. When viruses were saturated with heparin prior to SPR analysis, binding to the Heparin chip was inhibited (data not shown).

The binding activities of the VSV-based pseudotyped viruses to NAdOS-H or dNS-H immobilized on SPR sensor chips (NAdOS-H or dNS-H chips, respectively) were also examined (middle and bottom columns of Figs. 4A and B). We found that VSV-based pseudotyped viruses showed dose-dependent binding to the NAdOS-H or dNS-

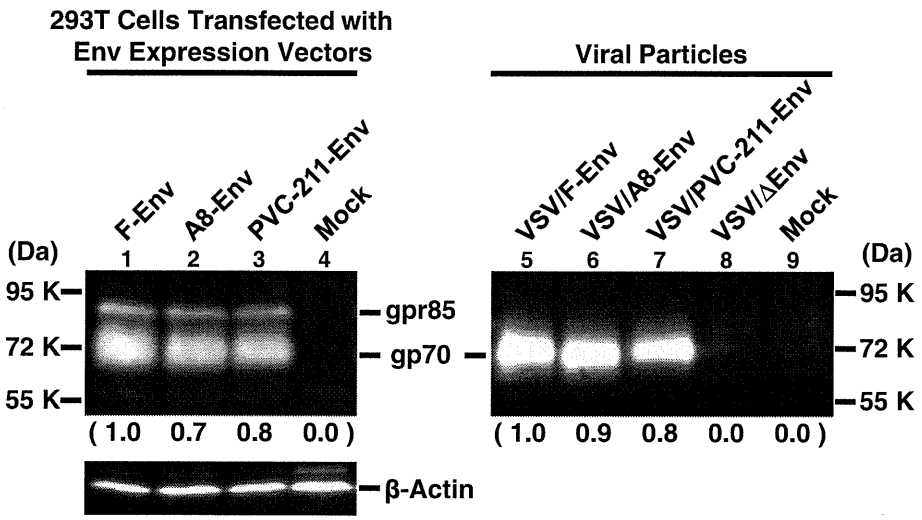

Fig. 2. Western blot analysis of the expression levels of Env in 293T cells transfected with Env expression vectors and VSV-pseudotyped virus particles. Lanes 1–3: 293T cells transfected with Env expression vectors. Lane 4: Mock-transfected 293T cells. Lanes 5–7: VSV-pseudotyped virus particles carring Env. 2×10^5 infectious units of viruses were applied to the Western blot. Details are given in the Materials and methods section. Lane 8: Env-deficient VSV-like particles prepared as described in the Materials and methods section. Lane 9: culture supernatant from 293T cells (used in the SPR analysis as a Mock control) was prepared as described in the Materials and methods section. To detect the Env protein, anti-SU (gp70) was used. Relative amount of Env protein is shown in parentheses. The expression levels of Env protein of transfected 293T cells were normalized against the intensity of the beta-actin band and are shown relative to the cell transfected with F-Env expression vector. The amount of Env protein in virions is shown relative to the VSV/F-Env. This figure is representative of repeat experiments. Experiments with each sample were performed twice and similar results were obtained.

H chips. The binding activities of VSV/A8-Env and VSV/PVC-211-Env to NAdOS-H or dNS-H were higher than those of VSV/F-Env. The binding activities of VSV/A8-Env and VSV/PVC-211-Env to NAdOS-H were clearly lower than those to heparin, but were similar or slightly lower than those to dNS-H. The binding activities of VSV/F-Env to NAdOS-H were slightly lower than those to heparin, and were not significantly different to those for dNS-H. Although NAdOS-H did not show any detectable inhibition of the infectivity of ecotropic MLV or VSV-based pseudotyped viruses carrying ecotropic Env, the binding activities of VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env to this ligand appeared to be comparable to those to dNS-H.

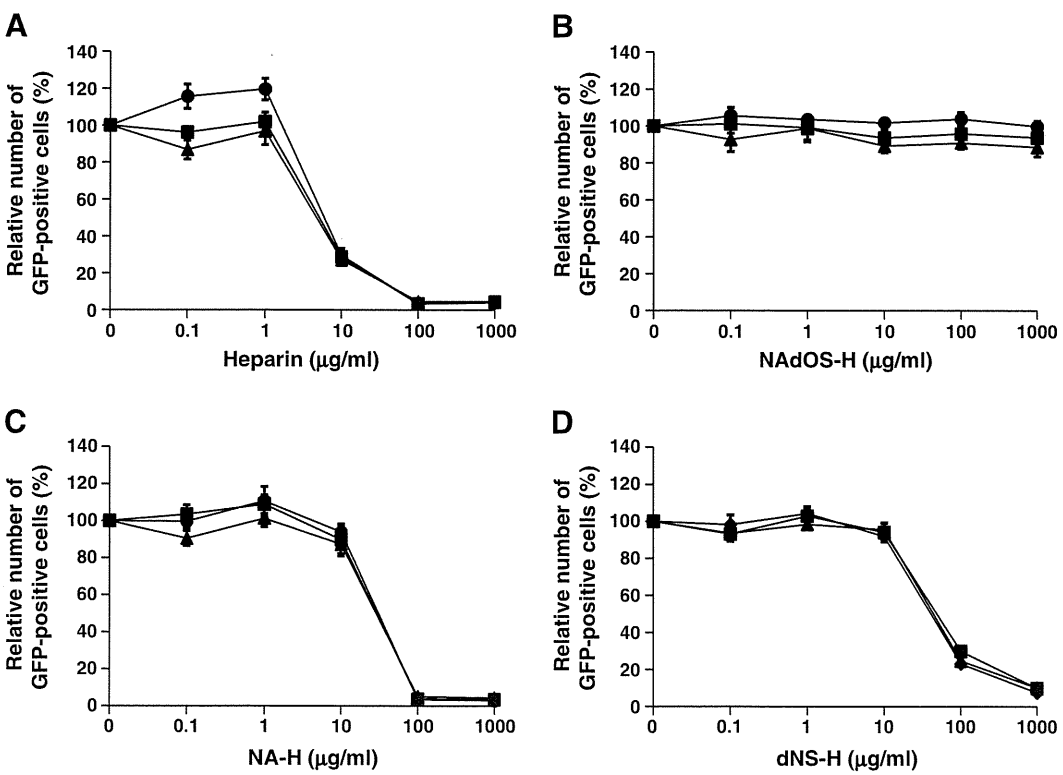

Fig. 3. Inhibitory activities of heparin and three derivatives against infection of VSV based-pseudotyped viruses carrying Env into NIH 3T3 cells. VSV/F-Env (triangle), VSV/A8-Env (circle), and VSV/PVC-211-Env (square) were pre-incubated with various concentrations of heparin (A), NAdOS-H (*N*-acetyl-de-*O*-sulfated heparin) (B), NA-H (*N*-acetylheparin) (C), or dNS-H (de-*N*-sulfated heparin) (D) for 1 h at 37 °C in the absence of polybrene. Then the virus–heparin mixture was inoculated onto NIH 3T3 cells in the presence of 10 μg/ml polybrene. After 16 h, GFP-positive cells were counted by FACS analyses. The mean values of 4 independent experiments and SEM are shown.