Biochemical and Biophysical Research Communications **287,** 42–46 (2001)
doi:10.1006/bbrc.2001.5534, available online at http://www.idealibrary.com on IDEAL®

Syndecan-4 as Antithrombin Receptor of Human Neutrophils

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Received August 8, 2001

Antithrombin inhibits chemokine-induced migration of neutrophils by activating heparan sulfate proteoglycan-dependent signaling. Mechanisms of antithrombin's effects on neutrophils were, therefore, studied by testing function and expression of heparan sulfate proteoglycans in RT-PCR or flow cytometry and cell migration assays, respectively. *In vitro* effects of antithrombin on human neutrophil migration in modified Boyden chambers were abolished by pretreating cells with heparinase-1, chondroitinase, sodium chlorate, and anti-syndecan-4 antibodies. Expression of syndecan-4 mRNA and protein in neutrophils was demonstrated in RT-PCR and anti-syndecan-4 immunoreactivity assay, respectively. In the presence of pentasaccharide, antithrombin lost its activity on the cells. Data suggest that antithrombin regulates neutrophil migration via effects of its heparin-binding site on cell surface syndecan-4.

Key Words: **heparan sulfate proteoglycans; chemotaxis; serpines; inflammation; sepsis; leukocyte; signaling; matrix.**

It has been shown that heparan sulfate proteoglycans (HSPG) from endothelium and leukocytes interact with P-selectin which is an important adhesion molecule regulating leukocyte adhesion and migration (1). HSPG localize to granules of myeloid cells including monocytes and neutrophils (2), and expression of mRNA for HSPG syndecans has been detected in monocytes/macrophages (3, 4)

Studies using intravital microscopy demonstrated that the serpin antithrombin (AT) attenuates ischemia-induced leukocyte extravasation and that thrombin plays an important role in this leukocyte recruitment response (5). It has been shown that AT is able to promote the release of prostacyclin from endothelial cells by interacting with HSPG at the endothelial cell surface *in vivo* (6), and also through binding to HSPG of human umbilical vein endothelial cells, AT is able to affect cell growth (7). In addition, high-affinity binding of AT to syndecans of smooth muscle cells has recently been confirmed (8). Recently, chemotactic effects of AT on neutrophils and signaling via interaction with cell surface HSPG have been reported (9).

Aim of the present study was to characterize the mechanisms by which AT regulates migration of neutrophils, which are involved in a variety of physiological and pathological conditions including inflammatory diseases of almost every organ system. We report that AT affects neutrophil migration via its heparin-binding site and action on cell surface syndecan-4.

MATERIALS AND METHODS

Leukocyte isolation. Neutrophils were prepared from forearm venous blood of healthy volunteers, anticoagulated with 1.6 mg EDTA/mL of blood. Neutrophil preparation by dextran sedimentation and hypotonic lysis of contaminating erythrocytes using sodium chloride solutions was performed as described previously (10).

AT preparations. Either the AT concentrate (c) KyberninP (Aventis Behring GmbH, Marburg, Germany) or a transgenic preparation of recombinant human (rh) AT (Genzyme, Framingham, MA) were used. One unit of AT (M_r 65 kDa) equals 23.1 μmol/L and is the activity present in 1 mL normal human pooled plasma tested in the presence of 0.1 U heparin.

Chemotaxis experiments. Neutrophil migration was measured using a modified 48-blindwell microchemotaxis chamber (Neuro Probe, Cabin John, MD) equipped with 5-μm pore-sized nitrocellulose filters (Sartorius, Goettingen, Germany). For chemotaxis 50 μL of the cell suspension [1×10^6 cells/mL] was put into the upper compartment of the chemotaxis chamber and cells were allowed to migrate for 30 min toward soluble chemoattractants in the lower wells. After this migration period, the filters were dehydrated, fixed and stained with hematoxylin–eosin. Migration depth was quantified by microscopy, measuring the distance from the surface of the filter to the leading front of three cells. Data are expressed as "chemotaxis index," which is the ratio between the distance of directed and undirected migration. Undirected migration (medium in upper and lower wells of the Boyden chamber) of neutrophils normally was between 45 μm and 60 μm.

When AT was used as attractant (9), 30 μL of AT at concentrations

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0006-291X/01 $35.00

ranging from 1 μU/mL up to 1 U/mL were filled in the lower wells and freshly prepared neutrophil suspension in the upper wells. For deactivation experiments, cells were incubated for 20 min with AT at concentrations ranging from 1 pU/mL to 10 U/mL and then washed twice before testing for chemotaxis. As positive control chemotactic agent in the lower chamber, interleukin-8 (IL-8) or formylmethionyl-leucylphenylalanine (fMLP) (both, Sigma Chemical Corp. (St. Louis, MO) were used.

For some experiments, cells were pretreated with heparinase I (Sigma Chemical Corp., St. Louis, MO), which is an enzyme that cleaves highly sulfated regions of heparan sulfate-like glycosaminoglycans (GAGs) at 2-O-sulfated uronic acids, for 50 min, washed twice and then used for direct chemotaxis and deactivation experiments. For other experiments cells were pretreated with chondroitinase ABC (Sigma Chemical Corp., St. Louis, MO) for 50 min that cleaves chondroitin sulfate side chains of cell surface GAGs. Since it is known that sodium chlorate is able to modify proteoglycan sulfation, we tested AT chemotaxis after a pretreatment of cells (20 min, two washing steps) with sodium chlorate (Merck, Darmstadt, Germany).

To block the heparin binding site on AT before its use in chemotaxis experiments, rhAT [1 U/mL] was incubated with the synthetic pentasaccharide SR90107A in concentrations ranging from 10 pmol/L to 100 nmol/L for 20 min at room temperature. As control, IL-8 [10 ng/mL] was incubated with the pentasaccharide. The suspensions were then put in the lower wells of the chemotaxis chambers and experiments were performed as described above. SR90107A (11) was a gift from Sanofi-Synthélabo Recherche (Toulouse, France).

As neutrophil migration might be mediated via syndecan-4, chemotaxis experiments were performed in the presence of monoclonal antibodies toward the core-protein of syndecan-4 and a side chain of this proteoglycan (both Santa Cruz Inc., Wiltshire, England). Cells were incubated with the antibodies for 20 min, washed twice and allowed to migrate toward AT.

FACS analysis. Fluorometric analysis for syndecan-4 expression was performed. A total of 5×10^5 cells were washed twice in PBS containing 0.5% BSA and incubated with 150 μg/mL human IgG for 20 min at 4°C. After pelleting, cells were incubated alternatively with 10 μg/mL anti-core syndecan-4 (Santa Cruz Inc., Wiltshire, England) or the respective isotype-matched control IgG (Sigma Chemical Corp., St. Louis, MO) for 30 min at 4°C. After washing, 10 μg/mL biotinylated goat anti-mouse IgG (PharMingen, Lexington, KY) was incubated for another 30 min. Cells were washed twice and subsequently, incubated with a 1:25 dilution of streptavidin-PE (Becton–Dickinson; San Jose, CA), washed twice and immediately analyzed on a FACS (Becton–Dickinson FACScan; San Jose, CA) with Cellquest software.

RT-PCR. Total RNA was isolated from 8×10^6 cells by phenol–chloroform–isoamyl alcohol extraction (RNAClean; Hybaid-AGS, Ulm, Germany). Reverse transcriptase reaction was performed on 1 μg of RNA using random hexamers reverse transcriptase (Gibco BRL, Life Technologies, Vienna, Austria). 10 μL of the reverse transcriptase reaction mixture was then subjected to 35 cycles of PCR in a 50 μL reaction mixture containing 1.0 pmol of sense and antisense primer pairs in a Perkin–Elmer thermocycler: 95°C for 30 s (denaturation), 57°C for 60 s (annealing), 72°C for 30 s (extension). Hot Start *Taq* polymerase was from Qiagen Inc. (Valencia, CA). Primers (MWG Biotech, Ebersdorf, Germany) were designed to amplify a 453-bp coding sequence of syndecan-4. Sense: CGA GAG ACT GAG GTC ATC GAC; antisense: GCG GTA GAA CTC ATT GGT GG. The PCR products were subjected to agarose gel analysis.

Endothelial cell culture. HUVEC from fresh placenta cords were isolated and grown to confluence in a humidified atmosphere at 37°C. The growth medium was ECGM supplemented with 10% FCS.

Statistical analysis. Data are expressed as mean and standard error of the mean (SEM). Means were compared by Mann–Whitney *U* test after Kruskal–Wallis analysis of variance. A P value < 0.05 was considered significant. Analyses were performed using the StatView software package (Abacus Concepts, Berkeley, CA).

RESULTS

Effects of heparinase I and chondroitinase on AT-induced migration of PBMC. To investigate the role of intact HSPG on the cell surface of neutrophils for AT-induced cell migration and chemotaxis deactivation as was shown previously (9), neutrophils were pretreated for 50 min with heparinase I [50 nU/mL to 50 mU/mL] or chondroitinase [50 nU/mL to 50 mU/mL] at 37°C, followed by washing. As glypicans carry heparan sulfate but not chondroitin sulfate side chains, whereas syndecans carry both (7), experiments were performed with heparinase I and chondroitinase in order to differentiate between the two HSPGs. Chemotactic effects of ATc [1 U/mL] (data not shown) or rhAT [1 U/mL] were found to be completely abolished by pretreatment with both, heparinase I and chondroitinase, whereas chemotactic effects of fMLP [10 nmol/L] remained unchanged (Fig. 1A).

Effects of antibodies to syndecan-4 on the chemotactic response of neutrophils to AT. Since AT-induced chemotaxis of neutrophils was inhibited by both chondroitinase and heparinase I, thus suggesting syndecan involvement, and as it is known that AT binds to syndecan-4 (12), chemotaxis experiments with AT were performed using monoclonal antibodies to syndecan-4 core protein and to a syndecan-4 side chain. Cells were pretreated with the two antibodies or isotype matched IgG, and then allowed to migrate toward rhAT [1 U/mL]. Antibodies to syndecan-4 core protein and to a side chain of syndecan-4 inhibited directed migration of neutrophils toward rhAT in a concentration dependent manner (Fig. 1B).

To investigate the effect of sodium chlorate which is known to modify sulfation of syndecans in cell culture (13), neutrophils were pretreated with sodium chlorate [10 mmol/L to 40 mmol/L] and after washing cells twice, rhAT-induced [1 U/mL] chemotaxis was tested. Neutrophil chemotaxis to rhAT was significantly inhibited by sodium chlorate whereas chemotaxis toward IL-8 was not affected (data not shown).

Effect of ligating the heparin-binding site on AT with the synthetic pentasaccharide SR90107A on migratory actions of AT. By blocking the pentasaccharide binding sequence on AT with SR90107A, the chemotactic activity of rhAT [1 U/mL] on neutrophils was inhibited. When IL-8 was coincubated with SR90107A, the chemotactic response of the cells toward IL-8 was not affected by SR90107A (Fig. 1D).

Expression of syndecan-4 core protein in neutrophils. Since AT-induced effects on chemotaxis of neutrophils may be mediated by its binding to and activation of

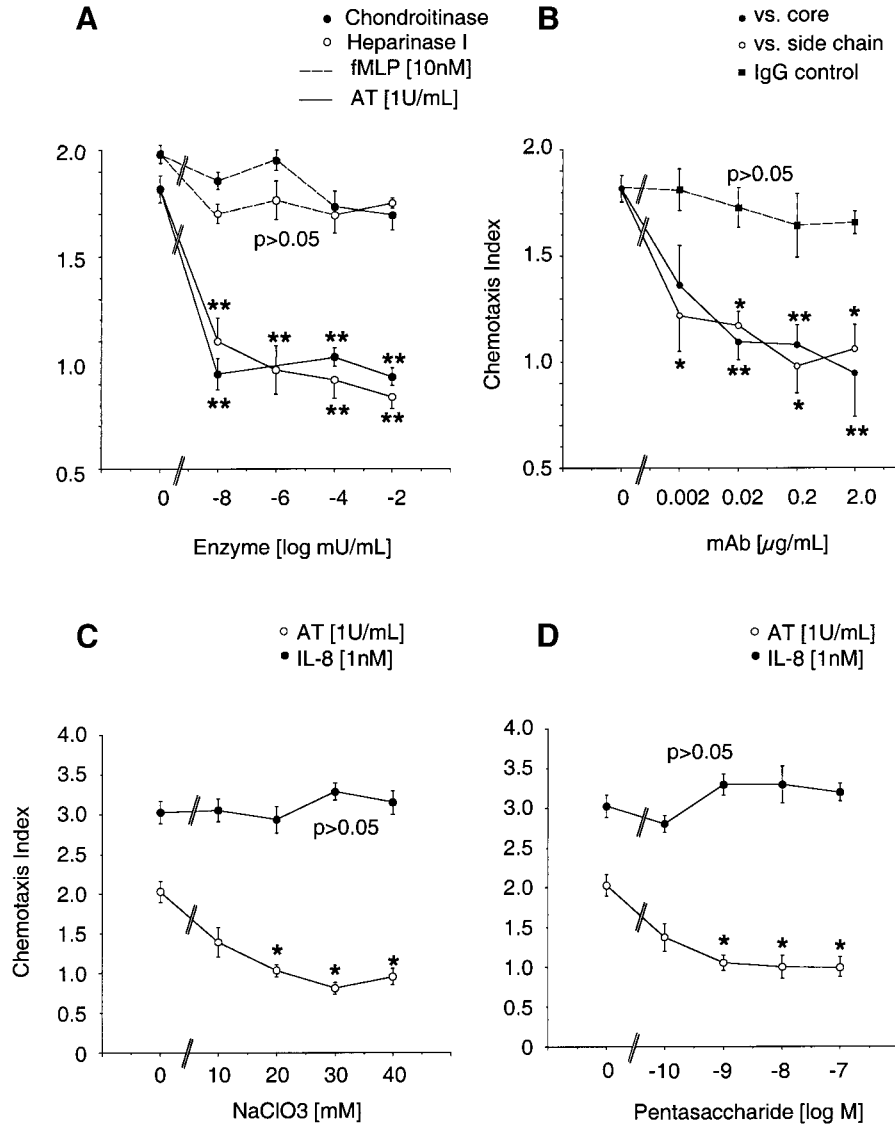

FIG. 1. Effects of heparinase, chondroitinase, syndecan-4 antibodies, sodium chlorate, and pentasaccharide on AT-induced chemotaxis of neutrophils. (A) Heparinase I or chondroitinase was added to neutrophils (37°C/5% CO_2). After an incubation period of 50 min, cells were washed twice and chemotaxis experiments were performed. AT [1 U/mL] served as chemoattractant and fMLP [10 nM] served as a control. Data are expressed as chemotaxis index which is the ratio between directed and undirected migration. Random migration was 56 ± 2.1 μm (mean ± SEM). Statistical analysis: Mann–Whitney U (*$P < 0.05$; **$P < 0.001$) after Kruskal–Wallis ($P < 0.01$); $n = 6$. (B) Preincubation of neutrophils with antibodies to syndecan-4 core protein and syndecan-4 side chain epitopes was performed for 20 min (37°C/5% CO_2). After washing cells were allowed to migrate toward AT [1 U/mL] in modified Boyden chambers using nitrocellulose micropore filters. Isotype-matched IgG served as control. Response is expressed as chemotaxis index (mean ± SEM), which is the ratio between directed and undirected migration of cells. Distance of undirected migration was 68 ± 4.9 μm ($n = 5$). Statistical analysis: Mann–Whitney U test (*$P < 0.05$; **$P < 0.001$) vs medium after Kruskal–Wallis ($P < 0.01$). (C) Cells were incubated with various concentrations of sodium chlorate [10 to 40 mM]. After washing cells were allowed to migrate toward AT [1 U/mL] or IL-8 [1 nM] as a control. Data are expressed as chemotaxis index. Statistical analysis: Mann–Whitney U test (*$P < 0.05$) vs medium after Kruskal–Wallis ($P < 0.01$); $n = 4$. (D) Coincubation of AT with different concentrations of SR90107A for 20 min at room temperature was performed before testing chemotaxis toward AT. Chemotaxis experiments were performed in modified Boyden chambers. As control attractant, IL-8 was coincubated with SR90107A. Results are given as mean ± SEM of migration index, which is the ratio between the distance of migration toward AT and that toward medium. Multiple group comparison was performed after Kruskal–Wallis ($P > 0.01$) followed by Mann–Whitney U test (*$P < 0.05$); $n = 4$.

syndecan-4, surface expression of syndecan-4 on these cells and its mRNA content were tested to confirm presence of this core protein in these cells. In FACS analysis, a slight but significant shift of fluorescence in neutrophils was observed by anti-syndecan-4 core antibody indicative of cell surface presence of syndecan-4 (Fig. 2A). To determine whether syndecan-4 mRNA is found in neutrophils, RT-PCR was performed. Though only a weak signal of the specific amplified product of 453 bp size was observed in several independent ex-

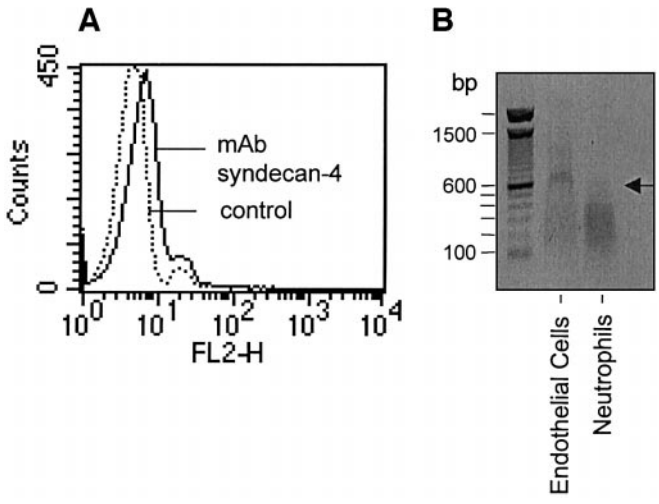

FIG. 2. FACS and RT-PCR analyses of syndecan-4 expression in neutrophils. (A) Fluorescence analysis used a FACScan flow cytometer, and a histogram of PE-fluorescence is shown. Cells were preincubated with 10 μg/mL isotype-matched control mouse IgG (thin line) or with 10 μg/mL anti-syndecan-4 mAb (bold line) and stained with PE-conjugated streptavidin. (B) For RT-PCR, 1 μg of total RNA from either neutrophils or human umbilical vein endothelial cells was reverse transcribed into cDNA and amplified for the syndecan-4 gene using PCR. Syndecan-4 is represented by the 453-bp product.

periments, data show that syndecan-4 mRNA can be found in neutrophils (Fig. 2B).

DISCUSSION

Previously, observations were made on AT actions on neutrophil migration (9). To verify specific activation by AT of HSPG, the most prevalent type of GAG, we employed the specificity of heparinase I to highly sulfated polysaccharide chains containing linkages to 2-O-sulfated α-L-idopyranosyluronic acid residues (14). Heparinase I has recently been described to degrade the synthetic heparin pentasaccharide to a disaccharide and trisaccharide product (15). Pretreatment of neutrophils with heparinase I abrogated the AT-induced migratory response of neutrophils as was reported before (9). In contrast, chemokine-induced effect on neutrophils was not affected by heparinase I. From this it was suggested that the pentasaccharide sequence of HSPG of neutrophils which is cleaved by heparinase I may important for mediating the effects of AT on the cells (9).

Heparan sulfate chains abound on syndecans and glypicans and can bind a diverse repertoire of proteins. Thereby, HSPG can immobilize the ligands, increase its local concentration, change its conformation, present it to a signaling receptor and enhance the formation of receptor-ligand signaling complexes (16). Conceivably, modifications of these HS chains could influence cell functions. By cleaving this specific site known to bind and respond to AT, chemotactic effects as well as deactivation in response to AT were abolished.

To further substantiate the role of GAGs in the response of neutrophils to AT, cells were purified and preincubated with medium containing the GAG sulfation inhibitor, sodium chlorate (17). Such pretreatment dose-dependently abolished responsiveness of the cells to AT, thus, further supporting a novel role of GAGs in controlling leukocyte functions.

Of the two families of membrane-bound HSPG, the syndecans, by containing mixtures of the two mayor types of GAG chains found in animal cells, namely heparan sulfate and chondroitin sulfate, exemplify hybrid proteoglycans. In contrast, the glypicans appear to contain only heparan sulfate chains (18, 19). To examine whether chondroitin sulfate proteoglycans act as putative cell receptors for AT, neutrophils were treated with chondroitinase before testing cell's migration. Data demonstrating that AT's effects on cell migration are sensitive to both heparinase I and chondroitinase suggest that syndecans mediate direct cellular actions of AT.

To explore the mechanisms involved in this action of AT, we ligated the heparin-binding sequence on AT by the synthetic pentasaccharide SR90107A before testing AT effects in chemotaxis experiments. Previously, binding of the pentasaccharide to AT was shown to induce a conformational change which results in an accelerated inhibition of blood coagulation factor Xa by AT (20) but this conformational change is insufficient to induce inhibition of thrombin by AT (21). Preincubation of AT with the pentasaccharide led to a complete disappearance of its chemotactic effect, thus highlighting the essential role of the pentasaccharide-binding sequence on AT.

Previously, members of the HSPG family were just seen as co-receptors but there is growing evidence that they have intrinsic signaling capacity. At present, maybe the best understood of them all is syndecan-4. It has been shown that the core protein cytoplasmic domain of syndecan-4 can signal during adhesion (22, 23) and its signaling properties have been extensively described (24–26).

Syndecan-4 has been proved to exist on B lineage lymphocytes (27) and on a variety of mature macrophage-like cells (3), but direct evidence for syndecan-4 core protein expression in neutrophils was still missing. Using FACS and RT-PCR analyses of syndecan-4 of neutrophils, we detected the presence of syndecan-4 in neutrophils. Finally, the observation that AT-affected migration of the cells was specifically inhibited by anti-syndecan-4 antibodies provides functional confirmation of the involvement of syndecan-4-typical in cellular effects of AT.

Until now, no specific receptor for AT has been identified in leukocytes, except for its interactions with GAGs on cell surfaces. Our results provide strong evidence for interaction of AT with heparan sulfate and chondroitin sulfate chains attached to proteoglycans on the surface of leukocytes leading to a previously unknown specific action of AT. Biochemical and func-

tional tests identify syndecan-4 as putative functional receptor for AT which in this context acts via its heparin-binding site. Syndecans are important participants in cell surface signaling and critical in controlling cell behavior. They modulate specific receptor interactions, accelerate the formation of proteinase-proteinase inhibitor complexes, and mediate interactions of the cell surface with several enzymes and structural proteins (28). The cell surface proteoglycan functions pertain to the transduction of signals that emanate from the continuous interplay between matrix components, growth factors, and proteinases (29) and as we show here, endogenous blood coagulation inhibitors.

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