Erratum

Syndecan-4 mediates antithrombin-induced chemotaxis of human peripheral blood lymphocytes

In the print version, the spelling of the last author’s name was incorrect. The correct spelling is Christian J. Wiedermann.
Syndecan-4 mediates antithrombin-induced chemotaxis of human peripheral blood lymphocytes and monocytes

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Summary
Antithrombin inhibits chemokine-induced migration of neutrophils by activating heparan sulfate proteoglycan-dependent signaling. Whether antithrombin affects migration of other types of leukocytes is not known. We investigated the effects of antithrombin on spontaneous and chemokine-triggered migration of lymphocytes and monocytes from human peripheral blood in modified Boydan chamber micropore filter assays. Lymphocyte and monocyte populations from human peripheral blood were purified using magnetic antibody cell sorting. The signaling mechanisms required for antithrombin-dependent migration were studied using signaling enzyme blockers. Expression of heparan sulfate proteoglycan core protein was studied by RT-PCR and flow cytometry. The antithrombins used were Kybernin®P from human plasma and a monoclonal-antibody-purified preparation from this plasma. Pretreatment of lymphocytes and monocytes with antithrombin inhibited chemotaxis toward optimal concentrations of interleukin-8 or Rantes (regulated upon activation normal T-cell expressed and activated) at concentrations of antithrombin as low as 10 nU/ml. In the absence of the chemokines, direct exposure of cells to gradients of antithrombin stimulated migration. Effects of antithrombin were abolished by pretreating cells with heparinase-1, chondroitinase, sodium chlorate and anti-syndecan-4 antibodies. Expression of syndecan-4 mRNA and protein in monocytes and lymphocytes was demonstrated in RT-PCR and anti-syndecan-4 immunoreactivity assays, respectively. In the presence of pentasaccharide, antithrombin lost its effect on cells. Data indicate that antithrombin directly inhibits chemokine-stimulated migration of monocytes and lymphocytes via the effects of its heparin-binding site on cell surface syndecan-4 by activation of protein kinase C and Rho signaling.

Key words: Heparan sulfate proteoglycan, Migration, Antithrombin, Pentasaccharide, Signaling

Introduction
Heparan sulfate proteoglycans (HSPG) 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 (Rapraeger, 1993). HSPG constitute two novel molecular families. The first family includes four syndecan-like integral membrane proteoglycans; the second is made up of two or more glypican-related integral membrane proteoglycans. Cell-surface proteoglycans function as part of the signals transduction pathway that result from the continuous interplay between matrix components, growth factors, and proteinases (David, 1993). HSPGs from endothelium and leukocytes interact with P-selectin, which is an important adhesion molecule regulating leukocyte adhesion and migration (Koenig et al., 1998). HSPG localize to granules of myeloid cells including monocytes and neutrophils (Parmley et al., 1983), and expression of mRNA for HSPG syndecans has been detected in monocytes/macrophages (Yeaman and Rapraeger, 1993a; Yeaman and Rapraeger, 1993b).

Studies using intravital microscopy demonstrated that antithrombin (AT) attenuates ischemia-induced leukocyte extravasation and that thrombin plays an important role in leukocyte recruitment (Ostrovsky et al., 1997). Whether this effect of AT is exclusively due to its ability to inhibit thrombin or whether it also involves additional activities of AT, including direct effects on HSPG-dependent adhesion and migration processes of leukocytes, is currently not known. AT promotes the release of prostacyclin from endothelial cells by interacting with HSPG at the endothelial cell surface in vivo (Uchiba et al., 1995). Through binding to the HSPG of human umbilical vein endothelial cells, AT is also able to affect cell growth (Mertens et al., 1992). In addition, high-affinity binding of AT to syndecans of smooth muscle cells has recently been confirmed (Cizmeci-Smith and Carey, 1997). Thus, via binding to HSPG, AT might be able to directly affect migratory properties of leukocytes. Recently, chemotactic effects of AT on neutrophils and signaling via interaction with cell-surface HSPG, possibly via syndecan 4, have been reported (Dunzendorfer et al., 2001).

The aim of this study was to explore, using AT as a potential HSPG ligand, the role of HSPG in regulating the migration of
monocytes and lymphocytes. These cells are involved in a variety of physiological and pathological conditions, including inflammatory diseases of almost every organ system. We report that AT affects mononuclear leukocyte migration via its heparin-binding site and activation of syndecan-4-dependent signaling.

Materials and Methods

Leukocyte isolation

Peripheral blood mononuclear cells (PBMC) and neutrophils were prepared from forearm venous blood of healthy volunteers, anticoagulated with 1.6 mg EDTA per mL of blood. After Lymphoprep® (Nycomed, Oslo Norway) density-gradient centrifugation, PBMC and neutrophils were collected and subjected to the preparation steps listed below.

PBMC were washed three times with normal saline (Fresenius Kabi, Graz, Austria). Positive selection of monocytes was performed by adding MACS colloidal superparamagnetic microbeads conjugated with anti-human CD14 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) to cooled, freshly prepared PBMC preparations in MACS buffer [PBS (PAA Laboratories, Linz, Austria) with 5 mmol/L EDTA and 0.5% bovine serum albumin (BSA; Dade Behring, Marburg, Germany)], according to the manufacturer’s instructions. Lymphocytes were selected using microbeads conjugated with anti-human CD3 and CD19 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells and microbeads were incubated for 15 minutes at 4-6°C. In the meantime, the separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) was positioned in the MACS magnetic field and washed with MACS buffer at room temperature. The cells were then washed with MACS buffer, resuspended, then loaded onto the top of the separation column. The eluent containing CD14+ or CD3- and CD19- cells was withdrawn. After removal of the column from the magnet, trapped monocytes (CD14+) or lymphocytes (CD3+ and CD19+) were eluted with six volumes of the cold MACS buffer, centrifuged, then resuspended in medium containing 0.5% BSA. Preparations yielded a cell purity of approximately 98%.

Neutrophil preparation by dextran sedimentation and hypotonic lysis of contaminating erythrocytes using sodium chloride solutions was performed as previously described (Dunzendorfer et al., 1997).

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 Endothelial Cell Growth Medium (PromoCell, Heidelberg, Germany) supplemented with 10% FCS (PAA Laboratories, Linz, Austria). Tissue-culture flasks and 96-well plates were coated with 0.2% gelatine (Sigma Chemical Corp., St. Louis, Missouri, USA) before seeding of cells. HUVeC that had been passaged twice were used for RNA extraction.

AT preparations

Either the AT concentrate Kybernin®P or its eluted immune-adsorbed fraction (Mab-purified AT), which was purified by running the concentrate over a monoclonal antibody resin (Aventis Behring GmbH, Marburg, Germany), was used in the chemotaxis experiments. One unit of AT (M₆₇, 65 kd) equals 23.1 µmOL/L and this is the activity present in 1 mL of normal human pooled plasma tested in the presence of 0.1 U heparin (Damus and Rosenberg, 1976).

Chemotaxis experiments

Leukocyte migration was measured using a modified 48 blindwell microchemotaxis chamber (Neuro Probe, Cabin John, Maryland) equipped with 5 µL pre-sized nitrocellulose filters (Sartorius, Goettingen, Germany). For chemotaxis, 50 µL of a cell suspension (1x10⁶ cells/mL) was put into the upper compartment of the chemotaxis chamber, and cells were allowed to migrate for 60 minutes (monocytes) or 90 minutes (lymphocytes) toward soluble chemoattractants in the lower wells. After these migration periods, the filters were dehydrated, fixed and stained with haematoxylin-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 a ‘chemotaxis index’, which is the ratio between the distance of directed and undirected migration.

To test if AT itself has chemotactic properties on PBMC, 30 µL of AT at concentrations ranging from 1 µg/mL up to 1 U/mL were added to the lower wells and freshly prepared lymphocyte or monocyte suspensions were added to the upper wells.

For deactivation experiments, cells were incubated for 20 minutes with Kybernin®P at concentrations ranging from 1 pU/mL to 10 µU/mL and then washed twice before testing for chemotaxis. As chemotactic agents for the lower chamber, either 10 ng/mL of Rantes (regulated upon activation normal T-cell expressed and secreted) or 1 nmol/L interleukin-8 (IL-8) was used for lymphocytes. For monocytes, either 10 ng/mL of Rantes or 10 nmol/L monocyte chemotactrant protein-3 (MCP-3) was used. (IL-8 was from Sigma Chemical Corp., St. Louis, Missouri; Rantes was from Pepro Tech LTD, London, England; MCP-3 was from R&D Systems, Minneapolis, Minnesota). Formylmethionylleucylphenylalanine (fMLP) was purchased from Sigma Chemical Corp. (St. Louis, Missouri).

For some experiments, cells were pretreated with heparinase I (Sigma Chemical Corp., St. Louis, Missouri, USA), which is an enzyme that cleaves highly sulfated regions of heparan-sulfate-like glycosaminoglycans (GAGs) at 2-0 sulfated uronic acids (Shriver et al., 2000), for 50 minutes, 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, Missouri), which cleaves chondroitin sulfate side chains of cell surface GAGs, for 50 minutes. Since sodium chlorate is able to modify proteoglycan sulfation, we tested AT chemotaxis after a pretreatment of cells with sodium chloride for 20 minutes (Merck, Darmstadt, Germany).

To block the heparin-binding site on AT before its use in chemotaxis experiments, AT [1 µU/mL] was incubated with the synthetic pentasaccharide SR90107A in concentrations ranging from 10 pmol/L to 100 nmol/L for 20 minutes at room temperature. As a control, Rantes [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 (Petitou et al., 1997) was a gift from Sanofi-Synthélabo Recherche (Toulouse, France).

In order to study the role of AT in controlling migration of leukocytes, cells were treated with 10 ng/mL of the intracellular enzyme blockers staurosporine (from streptomyces sp.), 500 nmol/L bisindolylmaleimide I GF 109203X (GFX), 10 nmol/L wortmannin (from penicillum fumiculosum), 10 µmol/L rolipram and 10 ng/mL thyrphostin 23 (all from Sigma Chemical Corp., St. Louis, Missouri).

As leukocyte migration might be mediated via syndecan-4 (Dunzendorfer et al., 2001), chemotaxis experiments were performed in the presence of antibodies against the core-protein of syndecan-4 (D-16) and the ectodomain of this proteoglycan (5G9) (both Santa Cruz Inc., Wiltshire, England). Cells were incubated with the antibodies for 20 minutes, washed twice and allowed to migrate toward the AT.

PBMC were also allowed to migrate toward gradients of thrombin (Sigma Chemical Corp., St. Louis, Missouri, USA) at various concentrations (100 µU/mL to 1 U/mL) for 60 minutes. Cells were also preincubated with thrombin (0.01 U/mL) with and without AT (1 U/mL). After washing twice, they then migrated.
toward Rantes (10 ng/mL). In another series of experiments, PBMC were first incubated with AT (1 μU/mL to 1 U/mL) followed by washing and then allowed to migrate towards thrombin (0.01 U/mL). These in vitro migration tests were performed to study the effects of the AT-binding site for thrombin on leukocytes. Random migration of monocytes was normally between 45 μm and 60 μm and that of lymphocytes was normally between 40 μm and 65 μm.

**FACS analysis**

Fluorometric analysis for syndecan-4 expression was performed on monocytes and lymphocytes. A total of 5x10⁶ cells were washed twice in PBS containing 0.5% BSA and incubated with 150 μg/mL human IgG for 20 minutes 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, Missouri, USA) for 30 minutes at 4°C. After washing, 10 μg/mL biotinylated goat anti-mouse IgG (PharMingen, Lexington, KY, USA) was incubated for another 30 minutes. Cells were washed twice, and monocytes and lymphocytes were subsequently incubated with a 1:25 dilution of streptavidin-PE (Becton-Dickinson; San Jose, CA), washed twice, then immediately analyzed on a FACS (Becton-Dickinson FACSscan; San Jose, CA) with Cellquest software.

**RT-PCR**

Total RNA was isolated from 8x10⁶ cells by phenol-chloroform-isooamylalcohol extraction (RNACleanTM; Hybaid-AGS, Ulm, Germany). A reverse transcriptase reaction was performed on 1μg 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 anti-sense primer pairs in a Perkin-Elmer thermocycler: 95°C for 30 seconds (denaturation), 57°C for 60 seconds (annealing) and 72°C for 30 seconds (extension). Hot Start Taq polymerase was purchased from Qiagen Inc. (Valencia, CA, USA). Primers (MWG Biotech, Ebersdorf, Germany) were designed to amplify a 453 bp coding sequence of syndecan-4. The sense primer sequence was CGA GAG ACT GAG GTC ATC GAC; The anti-sense primer sequence was GCG GTA GAA CTC ATT GGT GG. The PCR products were subjected to agarose gel analysis.

**Fig. 1.** Direct chemotactic effects of immunopurified AT on monocytes and lymphocytes. Chemotaxis experiments were performed in modified Boyden chambers using nitrocellulose micro pore filters. Monocyte chemoattractant protein 3 (MCP-3) for monocytes (left panel) and interleukin-8 (IL-8) for lymphocytes (right panel) were used as positive control chemotactic stimuli. Results are given as the mean±s.e.m. of the migration index, which is the ratio of the distance of migration (μm) towards a chemotactic agent and the distance (μm) towards the medium. Similar results were obtained using AT concentrate (data not shown). Multiple group comparison was performed after Kruskal-Wallis (p=0.0074 for lymphocytes, p=0.0001 for monocytes) followed by Mann-Whitney U-test (*, p<0.05). n=8 for lymphocytes, n=5 for monocytes.

**Statistical analysis**

Data are expressed as mean and standard error of the mean (s.e.m.). 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, California).

**Results**

Chemotaxis of PBMC, lymphocytes and monocytes toward different concentrations of AT

To explore the chemotactic properties of AT, freshly prepared PBMC and isolated monocyte and lymphocyte preparations were allowed to migrate towards different concentrations of a commercial AT concentrate that is in clinical use (Kybernin®P) or a monoclonal-antibody-purified AT. Concentrations ranging from 1 U/ml to 10 μU/ml significantly increased migration of all cells tested – PBMC (data not shown), lymphocytes and monocytes (Fig. 1). Rantes or IL-8, or Rantes or MCP-3 were used as positive controls for lymphocytes and monocytes, respectively. Checkerboard analyses revealed the migratory responses as true AT-induced chemotaxis defined as concentration-gradient-dependent migration (data not shown).

Inactivation of chemokine-induced chemotaxis of lymphocytes and monocytes by pretreatment with AT

Preincubation of lymphocytes and monocytes with various concentrations of AT concentrate was performed to ascertain interferences with chemokines and their migratory effects on PBMC. Migration of cells towards specific CC- and CXC-chemokines was deactivated by AT pretreatment. Lymphocyte chemotaxis towards Rantes was decreased significantly by pretreatment with Kybernin®P at concentrations from 1 U/ml to 10 μU/ml; chemotaxis towards IL-8 decreased at a similar concentration range but with less maximal activity (Fig. 2A). Chemotaxis of monocytes towards Rantes and MCP-3 mimicked the results obtained with lymphocytes, with Kybernin®P being a more potent deactivator of chemotaxis toward Rantes than towards MCP-3 (Fig. 2B).
Rantes; p=0.0038 toward MCP-3; toward Rantes; p=0.0163 toward IL-8; monocytes: p=0.0104 toward group comparison by Kruskal-Wallis test; lymphocytes: p=0.0197 Mann-Whitney U-test versus medium preincubation after multiple index, which is the ratio of the distance of migration (\(m\)) towards the medium. Results are given as the mean±s.e.m. of the migration monocyte chemoattractant protein 3 (MCP-3) \([10 \text{ nmol/L}]\) was chemotaxis of lymphocytes (A) toward Rantes \([10 \text{ ng/mL}]\) or IL-8 concentrations for 20 minutes followed by washing. Then micropore filters. Cells were preincubated with varying A T performed in modified Boyden chambers using nitrocellulose lymphocytes (A) and monocytes (B) by A T. Experiments were index, which is the ratio between directed and undirected migration. Random migration was 45±3.3 \(\mu\text{m}\) for monocytes and 56±2.1 \(\mu\text{m}\) for lymphocytes (mean±s.e.m.). For statistical analysis the Mann-Whitney U test was used (*, p<0.05) after the Kruskal-Wallis test (p<0.01); \(n=3\).

Effects of heparinase and chondroitinase treatment on A T-induced chemotaxis. Heparinase I or chondroitinase was added to monocytes or lymphocytes (37°C/5%CO\(_2\)). After an incubation period of 50 minutes, cells were washed twice and chemotaxis experiments were performed. A T [1 \(\text{U/mL}\)] served as chemotactrant. Data are expressed as chemotaxis index, which is the ratio of HSPGs. Chemotactic effects of A T were found to be completely abolished by pretreatment with both, heparinase I and chondroitinase (Fig. 3), whereas chemotactic effects of fMLP on monocytes or of IL-8 on lymphocytes remained unchanged (data not shown).

Presence of heparinase I during preincubation of monocytes with A T prevented the deactivation of chemotactrant-induced migration compared to preincubation with A T alone. The chemotaxis index of medium-treated monocytes toward fMLP was 1.8±0.11; pretreatment of monocytes with A T decreased this chemotaxis index to 1.3±0.10, whereas coincubation with heparinase I and A T restored chemotaxis to an index of 1.7±0.10 (Kruskal Wallis test p<0.05; \(n=4\)). For lymphocyte chemotaxis induced by IL-8, the corresponding values were 2.0±0.18 (medium treated), 1.3±0.07 (AT treated) and 1.9±0.13 (AT and heparinase I treated). Deactivation through A T and the restoration of deactivation after heparinase treatment were statistically significant (p<0.05, \(n=4\)).

**Fig. 2.** Deactivation of chemokine-induced migration of lymphocytes (A) and monocytes (B) by AT. Experiments were performed in modified Boyden chambers using nitrocellulose micropore filters. Cells were preincubated with varying AT concentrations for 20 minutes followed by washing. Then chemotaxis of lymphocytes (A) toward Rantes [10 \(\text{ng/mL}\)] or IL-8 [1 \(\text{nmol/L}\)] and of monocytes (B) toward Rantes [10 \(\text{ng/mL}\)] and monocyte chemoattractant protein 3 (MCP-3) [10 \(\text{nmol/L}\)] was monitored. Results are given as the mean±s.e.m. of the migration index, which is the ratio of the distance of migration (\(\mu\text{m}\)) towards attractant and the distance (\(\mu\text{m}\)) towards the medium. *, p<0.05. Mann-Whitney U-test versus medium preincubation after multiple group comparison by Kruskal-Wallis test; lymphocytes: \(p=0.0197\) toward Rantes; \(p=0.0163\) toward IL-8; monocytes: \(p=0.0104\) toward Rantes; \(p=0.0038\) toward MCP-3; \(n=5\).

**Fig. 3.** Effects of heparinase and chondroitinase treatment on AT-induced chemotaxis. Heparinase I or chondroitinase was added to monocytes or lymphocytes (37°C/5%CO\(_2\)). After an incubation period of 50 minutes, cells were washed twice and chemotaxis experiments were performed. AT [1 \(\text{U/mL}\)] served as chemotactrant. Data are expressed as chemotaxis index, which is the ratio between directed and undirected migration. Random migration was 45±3.3 \(\mu\text{m}\) for monocytes and 56±2.1 \(\mu\text{m}\) for lymphocytes (mean±s.e.m.). For statistical analysis the Mann-Whitney U test was used (*, p<0.05) after the Kruskal-Wallis test (p<0.01); \(n=3\).

**Effects of heparinase I and chondroitinase on AT-induced migration of PBMC**

In order to investigate the role of intact HSPG on the cell surface of monocytes and lymphocytes for AT-induced cell migration and chemotaxis deactivation, monocytes or lymphocytes were pretreated for 50 minutes with heparinase I or chondroitinase at 37°C, followed by washing. As glypicans carry heparan sulfate but not chondroitin sulfate side chains, whereas syndecans carry both (Mertens et al., 1992), experiments were performed with heparinase I and chondroitinase in order to differentiate between the two HSPGs. Chemotactic effects of AT were found to be completely abolished by pretreatment with both, heparinase I and chondroitinase (Fig. 3), whereas chemotactic effects of fMLP on monocytes or of IL-8 on lymphocytes remained unchanged (data not shown).

Presence of heparinase I during preincubation of monocytes with AT prevented the deactivation of chemotactrant-induced migration compared to preincubation with AT alone. The chemotaxis index of medium-treated monocytes toward fMLP was 1.8±0.11; pretreatment of monocytes with AT decreased this chemotaxis index to 1.3±0.10, whereas coincubation with heparinase I and AT restored chemotaxis to an index of 1.7±0.10 (Kruskal Wallis test p<0.05; \(n=4\)). For lymphocyte chemotaxis induced by IL-8, the corresponding values were 2.0±0.18 (medium treated), 1.3±0.07 (AT treated) and 1.9±0.13 (AT and heparinase I treated). Deactivation through AT and the restoration of deactivation after heparinase treatment were statistically significant (p<0.05, \(n=4\)).

**Effect on the migratory actions of AT of ligating the heparin-binding site on AT with the synthetic pentasaccharide SR90107A**

By blocking the pentasaccharide-binding sequence on AT with SR90107A, the chemotactic activity of AT on lymphocytes and monocytes was inhibited. At concentrations of SR90107A ranging from 1 \(\text{nM}\) up to 100 \(\text{nM}\), chemotactic effects of AT disappeared. Only at concentrations of 10 \(\text{pmol/L}\) or lower SR90107A was unable to block AT's chemotactic activity. As a control experiment, Rantes was coincubated with SR90107A, but the chemotactic response of the cells toward Rantes was not affected by SR90107A (Fig. 4).

**Effects of AT on thrombin-induced migration of PBMC**

To verify that thrombin does not interfere with AT's effects on PBMC and to assess whether the thrombin-binding site of AT
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may be involved in its effects on migration, preincubation experiments of PBMC with 0.01 U/mL of thrombin were performed in the absence or presence of 1 U/mL AT. No effect of thrombin on AT-induced deactivation of PBMC chemotaxis toward Rantes was seen. When cells were first exposed to AT and thrombin and then allowed to migrate toward Rantes, AT deactivated chemotaxis, whereas thrombin did not (Fig. 5A). Thrombin itself induced migration of PBMC (Fig. 5B), and preincubation with AT [1 μU/mL to 1 U/mL] did not deactivate thrombin-induced PBMC chemotaxis (Fig. 5C).

Expression of syndecan-4 in lymphocytes and monocytes

As AT-induced effects on chemotaxis of monocytes and lymphocytes may be mediated by its binding to and activation of syndecan-4, surface expression of syndecan-4 on these cells and its syndecan-4 mRNA content were tested to confirm the presence of its core protein in these cells. Using FACS analysis, a slight but significant shift of fluorescence in lymphocytes and monocytes was observed by an anti-syndecan-4 antibody, which is indicative of the cell-surface presence of syndecan-4 (Fig. 6A). To determine whether syndecan-4 mRNA is found in monocytes and lymphocytes, RT-PCR was performed. Data confirm that syndecan-4 mRNA was found in human peripheral blood lymphocytes and monocytes as well as in endothelial cells that were used for control (Fig. 6B).

Effects of antibodies to syndecan-4 on the chemotactic response of PBMC to AT

Since AT-induced chemotaxis of monocytes and lymphocytes was inhibited by both chondroitinase and heparinase I, thus suggesting syndecan involvement, and as AT binds to syndecan-4 (Carey, 1997), chemotaxis experiments with AT were performed using antibodies to syndecan-4 (D-16 and 5G9). Cells were pretreated with the two antibodies or isotype-matched IgG and then allowed to migrate toward AT. Antibodies to the syndecan-4 core protein and to the ectodomain of syndecan-4 inhibited directed migration of lymphocytes and monocytes toward AT in a concentration-dependent manner. Rantes-induced chemotaxis was not influenced by pre-treatment with these antibodies (Fig. 7).

Effects of sodium chlorate on AT-induced migration of monocytes and lymphocytes

To investigate the effect of sodium chlorate, which is known to modify sulphation of syndecans in cell culture (Lemansky and Hasilik, 2001), monocytes and lymphocytes were pretreated with sodium chlorate [10 mmol/L to 40 mmol/L], and, after washing cells twice, AT-induced [1 U/mL] chemotaxis was tested. Monocyte and lymphocyte chemotaxis towards AT was significantly inhibited by sodium chlorate, whereas chemotaxis toward Rantes was not affected (Table 1).

Fig. 4. Effect of blocking of the heparin binding site on AT by SR90107A, a synthetic pentasaccharide, on AT-dependent PBMC migration. Coincubation of AT with different concentrations of SR90107A for 20 minutes at room temperature was performed before testing direct lymphocyte (A) and monocyte (B) chemotaxis towards AT. Chemotaxis experiments were performed in modified Boyden chambers. As a control attractant, Rantes was coincubated with SR90107A. Results are given as the mean±s.e.m. of the migration index, which is the ratio of the distance of migration towards AT and the distance towards the medium. Multiple group comparison was performed after Kruskal-Wallis (p=0.0153 for lymphocytes, p=0.0085 for monocytes) followed by Mann-Whitney U-test (*, p<0.05); n=4.
Blocking of intracellular signaling enzymes in AT-induced chemotaxis of monocytes and lymphocytes

To elucidate signaling pathways involved in transmitting AT effects in PBMC, different intracellular enzyme blockers were used and compared with effects in AT-induced migration of neutrophils (Dunzendorfer et al., 2001). Lymphocytes, monocytes and neutrophils were freshly isolated from the same donor. The blockers staurosporine, GFX, tyrphostin-23, wortmannin and rolipram were used at established signal-blocking concentrations but showed no detectable influence on random basal migration of the cells. Rolipram, a selective inhibitor of cAMP-specific phosphodiesterase, was the only blocker that was able to significantly diminish AT-induced chemotaxis in all three cell types. Staurosporine, which is a non-specific inhibitor of PKC that also affects protein kinase A signaling, decreased AT-induced chemotaxis in neutrophils but had no effect on AT-induced migration in lymphocytes or monocytes. The specific PKC inhibitor bisindolylmaleimide (GFX) significantly inhibited AT-induced migration only in neutrophils and in lymphocytes; the use of wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase, significantly reduced chemotaxis towards AT in lymphocytes and monocytes but had no such effect in neutrophils. The protein tyrosine kinase inhibitor tyrphostin-23 diminished AT activity in monocytes’ chemotaxis but not in lymphocytes’ activity (Table 2). These results indicate that in their migratory responses to AT, different leukocyte populations have different sensitivities towards various signaling-enzyme blockers.

Table 1. Effect of sodium chlorate on monocyte and lymphocyte migration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Monocytes (Chemotaxis index ± s.e.m.)</th>
<th>Lymphocytes (Chemotaxis index ± s.e.m.)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.96±0.195</td>
<td>1.70±0.242</td>
</tr>
<tr>
<td>Sodium chlorate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mmol/L</td>
<td>1.31±0.060*</td>
<td>1.23±0.284</td>
</tr>
<tr>
<td>20 mmol/L</td>
<td>1.48±0.090*</td>
<td>1.07±0.062*</td>
</tr>
<tr>
<td>30 mmol/L</td>
<td>1.11±0.076*</td>
<td>0.97±0.066*</td>
</tr>
<tr>
<td>40 mmol/L</td>
<td>0.83±0.041*</td>
<td>1.07±0.042*</td>
</tr>
</tbody>
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p-value:<br> <0.05 <0.05 <0.05 <0.05 <0.05

Cells were incubated with sodium chlorate for 20 minutes, washed twice and allowed to migrate toward AT (1 U/mL) or Rantes (10 ng/mL) for 60 minutes (monocytes) or 90 minutes (lymphocytes). Chemotaxis was performed in modified Boyden chambers. Data are expressed using the chemotaxis index, which is the ratio between directed and undirected migration. Statistical analysis used the Mann Whitney U test *, p<0.05 versus control; Kruskal Wallis test †, p<0.05; n=3.
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Discussion

The way AT exerts anticoagulatory effects, that is, functions as a serpin, is well studied as is its conformational activation via binding to a specific pentasaccharide sequence of heparan sulfate (Carrell, 1999). Less well characterized are mechanisms of anti-inflammatory effects of AT. Results presented here provide better insight into the cellular effects of AT in PBMC.

Anti-inflammatory effects of AT described so far are mostly attributable to a promotion of endothelial release of prostacyclin (Uchiba et al., 1996): administration of prostacyclin mimicked some of the effects of AT and indomethacin pretreatment antagonized activities of AT. Similar conclusions were drawn recently by Hoffmann and co-workers (Hoffmann et al., 2000), who observed a prostacyclin-mediated reduction of leukocyte adhesion by AT in an endotoxemia model in vivo. Others (Okada et al., 1999) reported that lung allograft rejection in their animal studies was affected by AT, and therefore they performed an in vitro assay on rat spleen T-cell proliferation. They identified AT as an inhibitor of T-cell proliferation stimulated by concanavalin A. So besides established prostacyclin-mediated effects, AT was also demonstrated to affect T-cell proliferation (Okada et al., 1999).

For studying direct effects of AT on leukocytes, we used an experimental in vitro system that employed isolated PBMC, lymphocytes and monocytes. Such an experimental set up excludes to a large extent indirect effects, for example, those mediated via thrombin antagonism of serpin-related AT action or endothelial prostacyclin release. As AT binds to the cell-

Fig. 6. FACS and RT-PCR analyses of syndecan-4 in lymphocytes and monocytes. (A) FACS analysis of anti-syndecan-4 mAb binding to lymphocytes and monocytes. Fluorescence analysis used a FACScan Flow cytometer, and a histogram of PE-fluorescence is shown. Cells were preincubated with either 10 \( \mu \text{g/mL} \) isotype-matched control mouse IgG (thin line) or 10 \( \mu \text{g/mL} \) anti-syndecan-4 mAb (bold line) and stained with PE-conjugated streptavidin. (B) Syndecan-4 mRNA in endothelial cells, lymphocytes and monocytes. 1 \( \mu \text{g} \) of total RNA from each sample was reverse transcribed into cDNA and amplified for the syndecan-4 gene using PCR. Syndecan-4 is represented by the 453 base pair product.

Fig. 7. Effects of syndecan-4 antibodies on AT-induced chemotaxis of monocytes and lymphocytes. Preincubation with antibodies to syndecan-4 core protein (D-16) and syndecan-4 ectodomain (5G9) was performed for 20 minutes (37°C/5% CO\(_2\)). After washing, cells were allowed to migrate toward AT [1 U/mL] or Rantes [10 ng/mL] in modified Boyden chambers using nitrocellulose micropore filters. Isotype-matched IgG served as control. Response of monocytes (A) and lymphocytes (B) is expressed using the chemotaxis index (mean±s.e.m.), which is the ratio between directed and undirected migration of cells. The distance of undirected migration was 68±4.9 \( \mu \text{m} \) for monocytes and 57±9.5 \( \mu \text{m} \) for lymphocytes (n=4). Statistical analysis was performed using the Mann-Whitney U test (*, p<0.05) versus medium after Kruskal-Wallis (p<0.01), n=4. Chemotaxis toward Rantes remained unaffected after pre-treatment with syndecan-4 antibodies (C). Kruskal-Wallis (p>0.1)
surface HSPG of endothelial cells and ligation with HSPG may not only alter endothelial cell functions but also that of peripheral blood neutrophils (Dunzendorfer et al., 2001). AT may affect PBMC via similar mechanisms. Furthermore, these cells are also known to express similar glycosaminoglycans (GAGs) (Parmley et al., 1983; Yeaman and Rapraeger, 1993a; Yeaman and Rapraeger, 1993b).

In the present study it was observed that AT by itself exerts chemotactic effects in lymphocytes and monocytes. Similar observations were made for AT actions on neutrophils (Dunzendorfer et al., 2001). Furthermore, AT is able to decrease chemokine-induced PBMC migration. Chemokines represent a large family of polypeptide mediators that are divided into subclasses on the basis of their conserved cysteine residues. For our experiments, we chose the CXC-chemokine IL-8 and the CC-chemokines Rantes and MCP-3 in order to specifically induce migration of the different leukocyte populations. Chemokines bind to G-protein-coupled seven transmembrane receptors, and for most chemokines distinct receptors have been identified (Wells et al., 1998). Nevertheless, there appears to be some redundancy both in the chemokines and their appropriate receptors and in their action on target cells (Mantovani, 1999). Thus, besides its specific receptor binding activity, chemokines bind to GAGs on leukocytes, and this binding may modify their chemotaxis (Kuschert et al., 1999). We observed that in lymphocytes as well as in monocytes Rantes-induced chemotaxis was antagonized by AT more potently than by IL-8- and MCP-3-induced chemotaxis. As it was found that Rantes can bind to GAGs with a higher affinity than IL-8 does (Kuschert et al., 1999), and AT-deactivated leukocyte chemotaxis toward Rantes more potently than towards IL-8, it may be that AT competes for chemokine binding to GAGs on cell surfaces and thereby affects Rantes more potently than it does IL-8. In support of such a hypothesis, the experiments of Burns and co-workers (Burns et al., 1998) can be mentioned. They showed that receptor activation by Rantes depends on cell-surface interactions between the C-terminal portion of Rantes and cell-surface GAGs. Other mechanisms of deactivation of chemokine-induced migration may include cross-deactivation at the post-receptor levels (Richardson et al., 2000).

In order to verify the specific binding of AT to 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 alpha-L-idopyranosyluronic acid residues (Desai et al., 1993). Heparinase I degrades the synthetic heparin pentasaccharide to a disaccharide and trisaccharide product (Yu et al., 2000). Pretreatment of monocytes or lymphocytes with heparinase I abrogated the migratory response of the leukocytes to direct exposure to AT as well as their deactivation of chemokine-induced migration. In contrast, chemotaxis induced by chemokines or deactivation induced by a combination of attractants other than AT was not affected by heparinase I. From this, it can be concluded that the pentasaccharide sequence of HSPG in monocytes and lymphocytes that is cleaved by heparinase I is important for mediating the effects of AT on the cells. Heparan sulfate chains abound on syndecans and glypicans and can bind to a diverse range of proteins. Therefore, HSPG can immobilize the ligands, increase its local concentration, change its conformation, present itself to a signaling receptor and enhance the formation of receptor-ligand signaling complexes (Bernfield et al., 1999). Conceivably, modifications of heparin sulfate chains might influence any of the functions mentioned above. By cleaving this specific site, which is known to bind and respond to AT, chemotactic effects and deactivation in response to AT were abolished.

To further substantiate the role of GAGs in the response of PBMC to AT, lymphocytes and monocytes were purified and preincubated with medium containing the GAG sulfation inhibitor, sodium chlorate (Fallgren et al., 2001). Such dose-dependent pretreatment abolished the responsiveness of the cells to AT, thus 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 major types of GAG chains found in animal cells, namely heparan sulfate and chondroitin sulfate, exemplify hybrid proteoglycans. In contrast, the glypicans contain only heparan sulfate chains (Salminen et al., 1996; Waldström and Ljungh, 1999). To examine whether chondroitin sulfate proteoglycans act as putative cell receptors for AT, monocytes and lymphocytes were treated with chondroitinase before testing each 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 novel action of AT, we ligated the heparin-binding sequence on AT to the synthetic pentasaccharide SR90107A before testing the effects of AT 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 (Choay et al., 1983), but this conformational change is insufficient to induce inhibition of thrombin by AT (Olson et al., 1992). 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.

The molar ratio of effective concentrations of AT and pentasaccharide in the experiments described suggests that SR90107A is able to functionally neutralize an excess of AT.
This may be explained by dissociation of the pentasaccharide after induction of a conformational change in AT that prevents it from being active on HSPG. Data indicate that AT affects PBMC locomotion via its pentasaccharide-binding sequence through cell-surface HSPG.

Previously, members of the HSPG family were just seen as co-receptors, but there is growing evidence that they have an intrinsic signaling capacity. At present, maybe the best understood of them is syndecan-4. The core protein cytoplasmic domain of syndecan-4 can signal during adhesion (Couch and Woods, 1999; Echtermeyer et al., 1999), and its signaling properties have been extensively described (Oh et al., 1997a; Oh et al., 1997b; Oh et al., 1998). There is some evidence indicating that syndecan-4 directly interacts with PKCa and with the phosphoinositide phosphatidylinositol 4,5-bisphosphate (PtdIns[4,5]P2) (Horowitz et al., 1999) as well as with Rho (Saoncella et al., 1999). Syndecan-4 exists on B lineage lymphocytes (Yamashita et al., 1999) and on a variety of mature macrophage-like cells (Yeaman and Rapaeger, 1993a), but direct evidence for syndecan-4 core protein expression in PBMC was still missing. Using RT-PCR and FACS analyses of syndecan-4 of monocytes and lymphocytes, we, therefore, first confirmed the presence of syndecan-4 in PBMC. 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 signaling of AT in PBMC.

Results from studies using various signaling-enzyme blockers confirm PKC-dependent signaling in neutrophils (Dunzendorfer et al., 2001) and favor PtdIns[4,5]P2-dependent signaling in PBMC. Interestingly, the selective PDE4 inhibitor rolipram attenuates AT-induced chemotaxis in all three leukocyte subpopulations. PDE4 dependence of lymphocyte chemotaxis toward IL-8 has previously been demonstrated (Hidi et al., 2000) and may thus not be specific for AT. Hence, different cell types show distinct patterns of signaling upon HSPG ligation with AT. Alternatively, different GAGs on leukocyte subpopulations may be involved in AT-induced signaling. However, further elucidation of the mechanisms involved is necessary to provide a better understanding of the signaling events elicited in AT-induced alteration of migration in different leukocyte populations.

Until now, no specific receptor/signaling pathway for AT has been described, except for an interaction 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 on immune cell populations. Biochemical and functional tests identify syndecan-4 as a putative functional receptor for AT, which in this context acts via its heparin-binding site. These observations may have some relevance in cell biology of leukocyte-matrix interactions.

References


