

Syndecan-4-dependent migration of human eosinophils

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Summary

Background Heparan sulphate proteoglycans (HSPGs) are important participants in cell surface signalling and critical in controlling cell behaviour. They modulate inflammatory cell maturation and activation, leucocyte rolling, adhesion to endothelium as well as extravasation and chemotaxis. Whether eosinophil's function is affected has not yet been reported.

Objective We investigated the effects of transgenic, recombinant anti-thrombin III and Kyberlin P[®], an anti-thrombin III concentrate, as HSPG ligands on spontaneous and chemokine-triggered migration of normal eosinophils from human peripheral blood in modified Boyden chamber micropore filter assays.

Methods Eosinophils from human peripheral blood were purified using magnetic antibody cell sorting. The signalling mechanisms required for anti-thrombin-dependent migration were studied using signalling enzyme blockers. Expression of HSPG core protein mRNA was studied by PCR.

Results Pre-treatment of eosinophils with anti-thrombin III inhibited chemotaxis toward optimal concentrations of eotaxin or RANTES (regulated upon activation normal T cell expressed and activated). In the absence of the chemokines, direct exposure to gradients of anti-thrombin III stimulated eosinophil migration. The effects of anti-thrombin III were abolished by pre-treating cells with heparinase-1, chondroitinase, sodium chlorate and anti-syndecan-4 antibodies. Syndecan-4 gene expression in eosinophils was confirmed in PCR. In the presence of pentasaccharide, anti-thrombin III lost its effect on the cells. Functional responses were also abrogated by inhibition of protein kinase C, phosphatidylinositol 3-kinase and phosphodiesterase.

Conclusion Data indicate that anti-thrombin III affects eosinophil motility via the effects of its heparin-binding site on cell surface syndecan-4. Ligation of syndecan-4 with anti-thrombin III induces eosinophil migration and deactivates motility toward chemokines. These observations suggest that syndecan-4-dependent signalling may control eosinophil locomotion.

Keywords anti-thrombin, eosinophils, heparan sulphate proteoglycan, migration, syndecan-4

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Introduction

Eosinophilic granulocytes are implicated in a variety of diseases including allergic disorders, vasculitic granulomatous diseases, immunological disorders, intestinal and pulmonary diseases, and not the least parasitic infections [1, 2]. Eosinophils are potent inflammatory cells that secrete a number of lipid mediators and proteins relevant to the pathophysiology of allergic diseases. Furthermore, eosinophils are also able to produce pro-inflammatory cytokines and thereby amplify the inflammatory reaction [3, 4].

Heparan sulphate proteoglycans (HSPGs) influence a multitude of molecules, cell types and processes relevant to inflammation [5]. 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 [6]. At the cell surface, proteoglycans of the syndecan family are the major source of heparan sulphates [7]. These highly conserved type I transmembrane HSPGs are expressed in a developmental and cell-type-specific pattern and participate in diverse biological processes ranging from morphogenesis to energy metabolism [8]. The serine protease inhibitor anti-thrombin III (AT III) is a potent anti-coagulant and also has remarkable anti-inflammatory properties, several of which result independent from its actions in the coagulation cascade [9]. The binding of AT III to a recently identified cellular receptor, syndecan-4, leads to interference with the intracellular signals induced by mediators like lipopolysaccharides and, thereby, to a down-modulation of the inflammatory response [10, 11].

The aim of the present study was to explore the role of syndecan-4 in regulating the migration of eosinophils by using AT III as a potential HSPG ligand. We report that AT III affects eosinophil migration via its heparin-binding site and activation of syndecan-4-dependent signalling.

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Materials and methods

Reagents

All stock solutions were stored at -20°C before use. RPMI 1640 with phenol red was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Bovine serum albumin (BSA) was from Dade Behring (Marburg, Germany). Dextran, heparinase I, chondroitinase ABC, staurosporine, bisindolylmaleimide, wortmannin, rolipram, tryphostin 23, hirudin and thrombin were from Sigma Chemical (St Louis, MO, USA). Kybernin P[®] was from Aventis Behring GmbH (Marburg, Germany) and sodium chlorate was from Merck (Darmstadt, Germany). Lymphoprep[®] was from Nycomed Pharma AS (Oslo, Norway). Dulbecco's phosphate-buffered saline (PBS) and L-glutamine were from PAA Laboratories (Linz, Austria), and Hanks' balanced salt solution (HBSS) without phenol red was from Invitrogen (Carlsbad, CA, USA). MACS separation columns and microbeads were from Miltenyi Biotec (Auburn, CA, USA). The microchemotaxis chambers were from Neuroprobe (Bethesda, MD, USA), and cellulose nitrate filters were from Sartorius (Goettingen, Germany). RNA-Bee was from Tel-Test Inc. (Friendswood, TX, USA), reverse transcriptase was from Gibco BRL (Life Technologies, Vienna, Austria), hot Star Taq polymerase was purchased from Quiagen Inc. (Valencia, CA, USA) and primers were from MWG Biotech (Ebersdorf, Germany). Certified[™] PCR agarose was from Bio-Rad (Hercules, CA, USA). SR90107A was a gift from Sanofi-Synthelabo Recherche (Toulouse, France). Syndecan-4 antibodies (D-16 and 5G9) were from Santa Cruz Inc. (Wiltshire, England). RNase-free DNase was from Promega Corporation (Madison, WI, USA).

Preparation of human eosinophils and neutrophils

Granulocytes were obtained from the peripheral blood of healthy donors with no history of atopic or hypereosinophilic conditions by dextran sedimentation and centrifugation through Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). This step was repeated once to remove mononuclear cells (MNCs); it was followed by hypotonic lysis of contaminating erythrocytes using sodium chloride solution. For eosinophil preparation, cells were resuspended in $50\ \mu\text{L}/5 \times 10^7$ cells ice-cold MACS buffer (PBS with 5 mmol/L EDTA and 0.5% BSA); an equal volume of MACS colloidal superparamagnetic microbeads conjugated with monoclonal anti-human CD16 mAb was added and incubated (30 min, 6°C). Recommended volumes of ice-cold MACS buffer were added to the cell/microbead mixture and the cell suspension was loaded onto the separation column. The eluate containing CD16⁺ eosinophils was collected, washed, resuspended in RPMI 1640/0.5% BSA, and the separation procedure was repeated to increase purity. The purity of sorted eosinophils was $>98\%$, as determined by morphology and fluorescence-activated cell sorting (FACS) analysis. Contaminating cells were $<1\%$ lymphocytes, $<1\%$ neutrophils and basophils, and negligible numbers of monocytes/macrophages.

Preparation of monocytes and lymphocytes

Peripheral blood MNCs were isolated from EDTA blood of healthy volunteers. After Lymphoprep[®] density gradient

centrifugation, peripheral blood MNCs were collected and washed three times with normal saline. Positive selection of monocytes or lymphocytes was performed by adding MACS colloidal superparamagnetic microbeads conjugated with monoclonal anti-human CD14 antibodies (monocytes) or monoclonal anti-human CD3 antibodies (lymphocytes) to cooled, freshly prepared peripheral blood MNC preparations in MACS buffer (PBS with 5 mM EDTA and 0.5% BSA) according to the manufacturer's instructions. Cells and microbeads were incubated for 15 min at $4-6^{\circ}\text{C}$. In the meantime the separation column was positioned in the MACS magnetic field and washed with MACS buffer at room temperature. The cells were washed with MACS buffer, resuspended, and loaded onto the top of the separation column. The eluent was withdrawn, and after removal of the column from the magnet, trapped monocytes (CD14⁺) or lymphocytes (CD3⁺) were eluted with a sixfold amount of cold MACS buffer, centrifuged, and resuspended in medium containing 0.5% BSA. Preparations yielded a purity of $\sim 98\%$.

Migration assay

Migration assays were performed using a modified 48-well Boyden microchemotaxis chamber (Neuroprobe) in which a $5\text{-}\mu\text{m}$ -pore-size cellulose nitrate filter separated the upper and the lower chamber. For chemotaxis, $50\ \mu\text{L}$ of a 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 (neutrophils), 60 min (eosinophils) or 90 min (monocytes) toward soluble chemoattractants in the lower wells. After the migration period, the nitrocellulose filters were dehydrated, fixed and stained with haematoxylin-eosin. The migration depth of the cells into the filters was quantified by microscopy, measuring the distance (μm) from the surface of the filter to the leading front of the cells. Data are expressed as a chemotaxis index (CI), which is the ratio between the distance of directed and undirected migration of cells into the nitrocellulose filters.

To test if AT III itself has chemotactic properties on eosinophils, $30\ \mu\text{L}$ of AT III at concentrations ranging from 10 nU/mL up to 1 U/mL were added to the lower wells and freshly prepared eosinophil suspensions were added to the upper wells. For deactivation experiments, cells were incubated for 20 min with Kybernin P[®] or a transgenic, recombinant AT III at concentrations ranging from 10 nU/mL to 1 U/mL and then washed twice with HBSS before testing chemotaxis. As chemotactic agents for the lower chamber, either RANTES (regulated upon activation normal T cell expressed and secreted) (10 ng/mL) or eotaxin (10 nmol/L) was used for the chemotaxis experiments.

In other experiments, cells were pre-treated with heparinase I, which is an enzyme that cleaves highly sulphated regions of heparan sulphate like glycosaminoglycans (GAGs) at 2-O-sulphated uronic acids [12], for 50 min, washed twice and then used for direct chemotaxis and deactivation experiments. Furthermore, cells were pre-treated with chondroitinase ABC, which cleaves chondroitin sulphate side chains of cell surface GAGs, for 50 min. Since sodium chlorate is able to modify proteoglycan sulphation, we tested AT III chemotaxis after a pre-treatment of cells with sodium chlorate for 20 min.

To block the heparin-binding site on AT III before its use in chemotaxis experiments, AT III (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 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.

To rule out possible interference of thrombin on AT III's effects on eosinophils, pre-incubation experiments of eosinophils were performed with thrombin (0.01 U/mL) and hirudin (1 U/mL) the absence or presence of AT III (1 U/mL). Moreover, chemotaxis experiments toward different concentrations of thrombin alone and after pre-incubation with AT III (1 µU/mL–1 U/mL) were performed.

Intracellular signalling of AT III on eosinophils was tested by pre-incubation of the cells with the intracellular enzyme blockers staurosporine (10 ng/mL) (from streptomyces sp.), bisindolylmaleimide I GF 109203X (GFX) (500 nmol/L), wortmannin (10 nmol/L) (from penicillium fusiculosum), rolipram (10 µmol/L) and tyrphostin-23 (10 ng/mL). As leucocyte migration might be mediated via syndecan-4, chemotaxis experiments were performed in the presence of antibodies toward the core protein of syndecan-4 (D-16) and the ectodomain of this proteoglycan (5G9). Cells were incubated with the antibodies for 20 min, washed twice and allowed to migrate toward AT III.

RT-PCR

Total RNA was isolated from 10^7 cells by an acid guanidinium thiocyanate–phenol–chloroform mixture. A reverse transcriptase reaction was performed on 1 µg of RNA using random hexamers reverse transcriptase. One microgram of the resulting cDNA was then subjected to 35 cycles of PCR in a 50 µL reaction mixture containing 1 pmol of sense and anti-sense primer pairs in a Biometra® thermocycler: 95 °C for 60 s (denaturation), 57 °C for 60 s (annealing) and 72 °C for 60 s (extension). Non-exon-spanning primers were designed to amplify a 453 bp coding sequence of human syndecan-4. The sense primer sequence was CGA GAG ACT GAG GTC ATC GAC. The anti-sense primer sequence was CCG GTA GAA CTC ATT GGT GG. The PCR products were subjected to agarose gel analysis. To exclude genomic DNA contamination, DNase digestion using RNase-free DNase (Promega Corporation) and PCR cycles using RNA without a reverse transcriptase reaction were performed.

Statistical methods

Data are expressed as mean $\pm$ SEM. Means were compared by the Mann–Whitney *U*-test, paired *t*-test and Kruskal–Wallis ANOVA. A difference with $P < 0.05$ was considered to be significant. Statistical analyses were performed using the StatView software package (Abacus Concepts, Berkeley, CA, USA).

Results

To explore chemotactic properties of AT III, freshly prepared eosinophils were allowed to migrate towards different

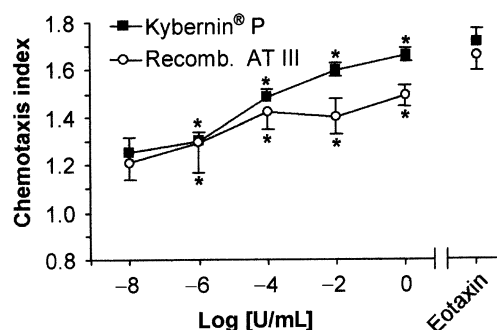


Fig. 1. Direct chemotactic effects of Kybernin P® (AT III) concentrate, and transgenic recombinant AT III on eosinophils. Chemotaxis experiments were performed in modified Boyden chambers using nitrocellulose micropore filters. As positive control chemotactic stimulus eotaxin (10 nmol/mL) was used. Results are given as mean $\pm$ SEM of migration index. Random migration was $91.5 \pm 1.89 \mu\text{m}$. Multiple group comparison was performed after Kruskal–Wallis followed by Mann–Whitney *U*-test (* $P < 0.05$); $n = 4$.

concentrations of a commercial AT III concentrate or a transgenic recombinant AT III. Concentrations ranging from 1 U/mL to 1 µU/mL significantly increased the migration of eosinophils (Fig. 1). Eotaxin was used as positive controls for eosinophil chemotaxis.

Pre-incubation of eosinophils with various dilutions of AT III concentrate was performed to ascertain its effects on chemokines and their migratory action on eosinophils. The migration of cells towards specific CC chemokines was deactivated by AT III pre-treatment. Eosinophil chemotaxis toward RANTES (10 ng/mL) and eotaxin (10 nmol/mL) was decreased significantly by pre-treatment with AT III concentrate (Fig. 2a) or a recombinant AT III (Fig. 2b) at concentrations from 1 U/mL to 100 µU/mL.

In order to investigate the role of intact HSPG on the cell surface of eosinophils for AT III-induced cell migration and chemotaxis deactivation, eosinophils were pre-treated for 50 min with heparinase I (50 nU/mL–50 mU/mL) or chondroitinase (50 nU/mL–50 mU/mL) at 37 °C, followed by washing. As glypicans carry heparan sulphate but not chondroitin sulphate side chains, whereas syndecans carry both [13], experiments were performed with heparinase I and chondroitinase in order to differentiate between the two HSPGs. Chemotactic effects of AT III (1 U/mL) were found to be completely abolished by pre-treatment with both heparinase I and chondroitinase (Fig. 3a), whereas chemotactic effects of RANTES (10 ng/L) on eosinophils remained unchanged (CI toward control 1.8 ± 0.05 ; pre-incubation with heparinase 1.7 ± 0.04 ; chondroitinase 1.7 ± 0.05 ; $P > 0.1$ each). The presence of heparinase I or chondroitinase during pre-incubation of eosinophils with AT III prevented the deactivation of chemoattractant-induced migration in a dose-dependent manner as compared with pre-incubation with AT III alone: the CI of medium-treated eosinophils toward RANTES was 1.62 ± 0.06 ; pre-treatment of eosinophils with AT III (1 U/mL) decreased this CI to 1.19 ± 0.04 , whereas co-incubation with heparinase I (500 mU/mL) or chondroitinase (500 mU/mL) and AT (1 U/mL) restored chemotaxis to an index of 1.57 ± 0.06 (heparinase) or 1.63 ± 0.05 (chondroitinase) (Kruskal–Wallis test $P < 0.05$; $n = 3$; Fig. 3b).

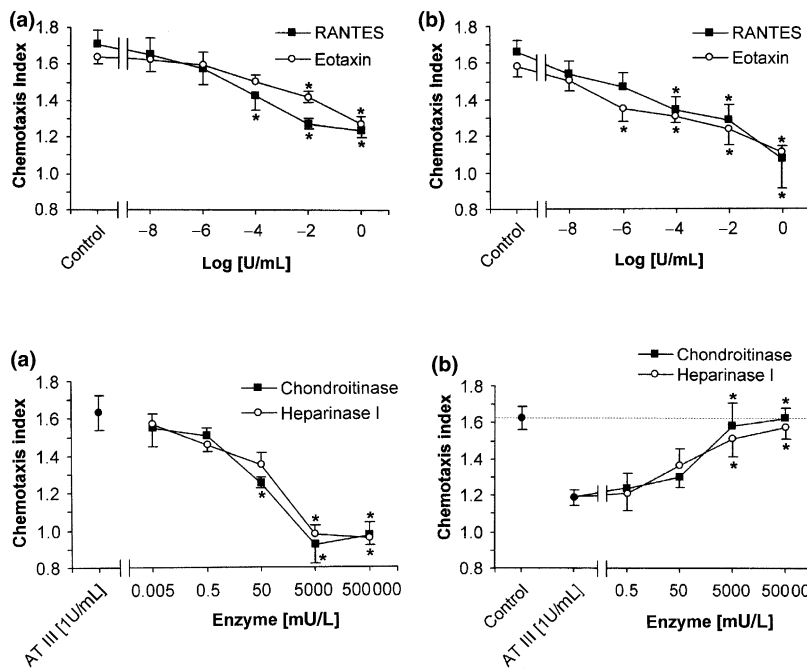


Fig. 2. Deactivation of chemokine-induced migration by Kyberlin P[®] and transgenic recombinant anti-thrombin III (AT III) on eosinophils. Chemotaxis experiments were performed in modified Boyden chambers using nitrocellulose micropore filters. Cells were pre-incubated with varying Kyberlin P[®] (a) and transgenic recombinant AT III concentrations (b) for 20 min followed by washing. Chemotaxis of eosinophils toward RANTES (10 ng/mL) or eotaxin (10 nmol/L) was performed. Results are given as mean $\pm$ SEM of migration index. Random migration was $92.78 \pm 2.20 \mu\text{m}$. Multiple group comparison was performed after Kruskal–Wallis followed by Mann–Whitney *U*-test ($*P < 0.05$); $n = 4$.

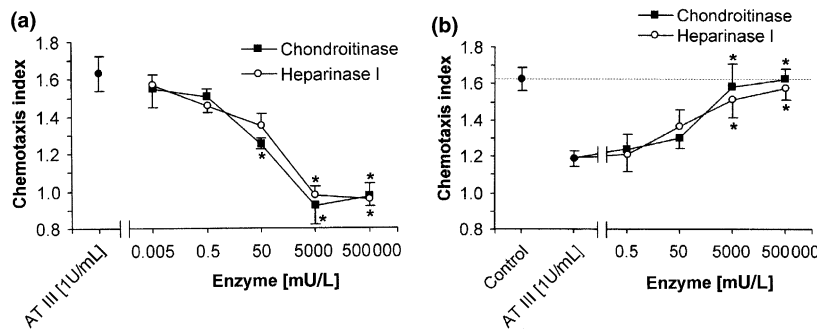


Fig. 3. Effects of heparinase and chondroitinase treatment on anti-thrombin III (AT III)-induced chemotaxis. (a) Eosinophils were pre-incubated with heparinase I or chondroitinase for 50 min (37°C/5% CO₂). After washing twice, migration toward AT III (1 U/mL) was tested. (b) Co-incubation of eosinophils with heparinase I or chondroitinase and an AT III (1 U/mL) concentrate. After an incubation period of 50 min (37°C/5% CO₂), cells were washed twice and chemotaxis toward RANTES (10 ng/mL) was performed. Data are expressed as chemotaxis index. Random migration was 96.92 ± 2.48 (mean $\pm$ SEM). Statistical analysis: Mann–Whitney *U*-test after Kruskal–Wallis ($*P < 0.05$); $n = 3$.

By blocking the pentasaccharide-binding sequence on AT III with SR90107A, the chemotactic activity of AT III (1 U/mL) on eosinophils was inhibited. At concentrations of SR90107A ranging from 1 μM up to 100 nM, chemotactic effects of AT III disappeared. As the control experiment, RANTES was co-incubated with SR90107A, yet chemotactic responses of the cells toward RANTES were not affected by SR90107A (Table 1).

To ensure that thrombin does not interfere with AT III's effects on eosinophils and to assess whether the thrombin-binding site of AT III may be involved in its effects on migration, pre-incubation experiments of eosinophils with thrombin (0.01 U/mL) were performed in the absence or presence of AT III (1 U/mL). When cells were first exposed to AT III and thrombin and then allowed to migrate toward RANTES (10 ng/mL), AT III alone and its combination with

thrombin deactivated chemotaxis whereas thrombin alone did not (Fig. 4a). Moreover, co-incubation of AT III (1 U/mL) and hirudin (1 U/mL), a direct thrombin inhibitor, had no influence on the migration-inhibitory effects of AT III. While Bolton et al. [14] demonstrated the presence of protease-activated receptor (PAR) 1 on the surface of eosinophils but could not show any effect of thrombin on eosinophil function, we found that thrombin is able to induce migration of eosinophils (Fig. 4b).

Since AT III-induced chemotaxis of eosinophils was inhibited by both chondroitinase and heparinase I, syndecan involvement is suggested. As it is known that AT III binds to syndecan-4 [15], which induces focal adhesion kinase and protein kinase C activation, chemotaxis experiments with AT III were performed using antibodies to syndecan-4, D-16 (Fig. 5a) and 5G9 (Fig. 5b). Cells were pre-treated with the two antibodies and then allowed to migrate toward AT III (1 U/mL). The antibodies, which are directed to syndecan-4 core protein (D-16) and to the ectodomain of syndecan-4 (5G9), inhibited directed migration of eosinophils toward AT III in a concentration-dependent manner. RANTES-induced chemotaxis was not influenced after pre-treatment with these antibodies.

To investigate the effect of sodium chlorate, which is known to modify sulphation of syndecans in cell culture [16], eosinophils were pre-treated with sodium chlorate (10–40 mmol/L) for 20 min and, after washing cells twice, AT III-induced (1 U/mL) chemotaxis was tested. Eosinophil chemotaxis to AT III was significantly inhibited by sodium chlorate whereas chemotaxis toward RANTES was not affected (Table 2).

To elucidate signalling pathways involved in transmitting AT III effects in eosinophils, different intracellular enzyme blockers were used and compared with effects in AT III-induced migration of neutrophils [10]. Lymphocytes, neutrophils and eosinophils were freshly isolated from the same

Table 1. Effect of blocking the heparin-binding site on AT III by SR90107A

	Chemotaxis index (mean $\pm$ SEM)	
	AT (1 U/mL)	RANTES (10 ng/mL)
Control	1.6 $\pm$ 0.08	1.7 $\pm$ 0.07
Pentasaccharide SR90107A		
1 $\mu\text{mol/L}$	1.1 $\pm$ 0.03*	1.7 $\pm$ 0.05
100 nmol/L	1.3 $\pm$ 0.07*	1.7 $\pm$ 0.08
10 nmol/L	1.4 $\pm$ 0.06*	1.6 $\pm$ 0.04
1 nmol/L	1.4 $\pm$ 0.05	1.7 $\pm$ 0.09
100 pmol/L	1.8 $\pm$ 0.03	1.7 $\pm$ 0.06

Exposure of AT III and RANTES to different concentrations of SR90107A for 20 min at room temperature was performed before testing eosinophil chemotaxis. Chemotaxis experiments were performed in modified Boyden chambers. Results are given as mean $\pm$ SEM of migration index. The distance of migration toward medium was $98.3 \pm 3.6 \mu\text{m}$ ($n = 3$). Multiple group comparison was performed after Kruskal–Wallis followed by Mann–Whitney *U*-test ($*P < 0.05$). RANTES, regulated upon activation normal T cell expressed and activated; AT III, anti-thrombin III.

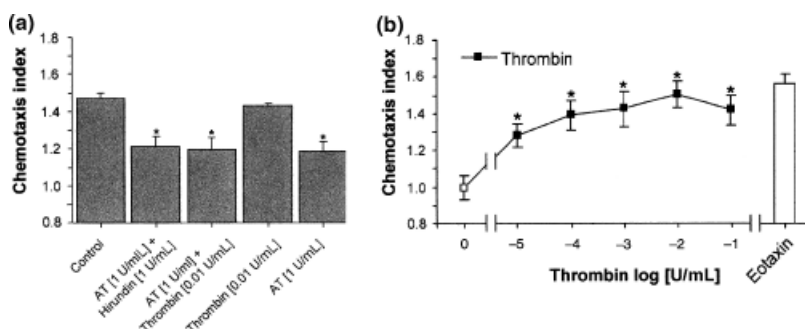


Fig. 4. Effect of thrombin on anti-thrombin III (AT III)-dependent eosinophil migration. Chemotaxis of eosinophils was tested in modified Boyden chambers. Data are expressed as chemotaxis index. (a) To test the effects of thrombin on AT III-induced deactivation of eosinophils chemotaxis toward RANTES (10 ng/mL), cells were pre-incubated in the presence of AT III with or without thrombin or hirudin for 20 min. Mann-Whitney *U*-test ($*P < 0.05$) vs. control after Kruskal-Wallis ($P < 0.01$); $n = 3$. (b) To test whether thrombin induces cell migration, eosinophils were allowed to migrate toward thrombin (100 μ U/mL–1 U/mL). Mann-Whitney *U*-test ($*P < 0.05$) vs. medium after Kruskal-Wallis ($P < 0.01$); $n = 3$.

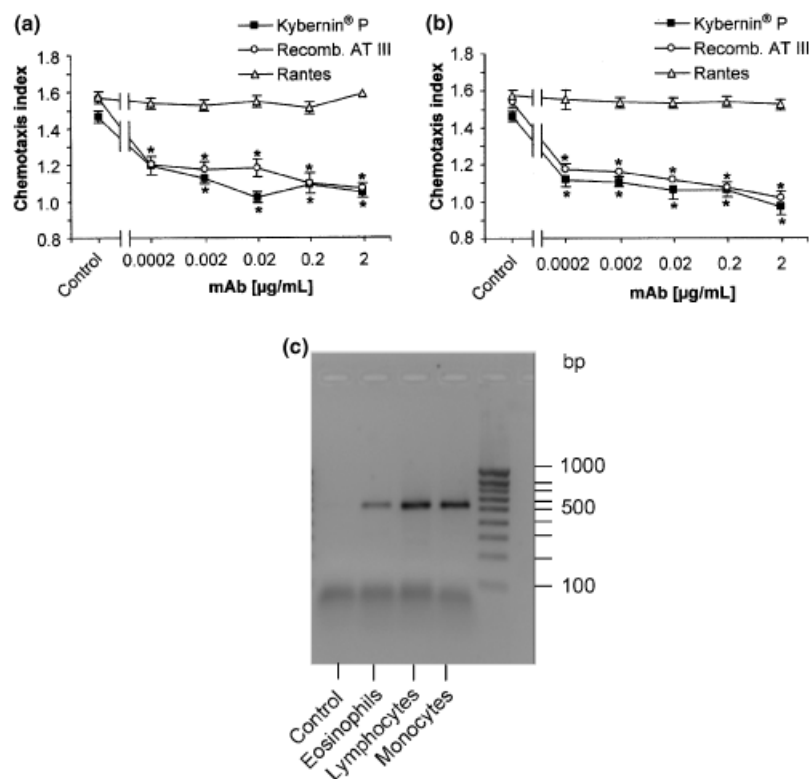


Fig. 5. Functional and molecular detection of syndecan-4 on human eosinophils. Pre-incubation with antibodies to syndecan-4 core protein (D-16) (a) and syndecan-4 ectodomain (5G9) (b) was performed for 20 min (37°C/5% CO₂). After washing, cells were allowed to migrate toward transgenic recombinant anti-thrombin III (AT III) (1 U/mL), AT III concentrate (1 U/mL) or RANTES (10 ng/mL) in modified Boyden chambers using nitrocellulose micropore filters. Response of eosinophils is expressed as chemotaxis index (mean $\pm$ SEM). The distance of undirected migration was $99.5 \pm 2.89 \mu$ m ($n = 3$). Statistical analysis: Mann-Whitney *U*-test ($*P < 0.05$) vs. medium after Kruskal-Wallis ($P < 0.01$), $n = 4$. (c) Syndecan-4 mRNA in eosinophils, lymphocytes and monocytes. One microgram 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 bp product. PCR minus any template was used as negative control.

donor. The blockers staurosporine, GFX, tyrphostin-23, wortmannin and rolipram were used at established signalling blocking concentrations but showed no detectable influence on basal random migration of the cells (data not shown). Rolipram, a selective inhibitor of cAMP-specific phosphodiesterase, and the specific PKC inhibitor bisindolylmaleimide (GFX) were the only blockers that were able to diminish AT III-induced chemotaxis significantly in all three cell types; staurosporine, which is a non-specific inhibitor of PKC that also affects protein kinase A signalling, decreased AT III-induced chemotaxis in eosinophils and neutrophils but had no effect on AT III-induced migration in lymphocytes. The use of wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase, significantly reduced chemotaxis towards AT III in eosinophils and lymphocytes, but had no such effect in neutrophils; the protein tyrosine kinase inhibitor tyrphostin-23 diminished AT III in eosinophil and lymphocyte chemotaxis but not in neutrophils (Table 3). These results indicate that in their migratory responses to AT III,

different leucocyte populations express different sensitivities towards various signalling enzyme blockers.

To determine whether syndecan-4 mRNA is expressed in eosinophils, RT-PCR was performed. Data show that syndecan-4 mRNA is present in human peripheral blood eosinophils as well as in lymphocytes and monocytes, which were used for control (Fig. 5). Control experiments excluded amplification of a genomic DNA contaminating the RNA samples.

Discussion

Chemotactic migration of eosinophils is a prerequisite for the cells to reach their site of biological action. Our working hypothesis is that AT III might modulate eosinophil chemotaxis directly via syndecan-4 and independent of other components of the coagulation cascade. The present study

Table 2. Effect of sodium chlorate on eosinophil migration

	Chemotaxis index (mean $\pm$ SEM)	
	AT (1 U/mL)	RANTES (10 ng/mL)
Control	1.6 $\pm$ 0.08	1.6 $\pm$ 0.06
Sodium chlorate		
10 nmol/L	1.0 $\pm$ 0.08*	1.5 $\pm$ 0.06
20 nmol/L	1.1 $\pm$ 0.03*	1.7 $\pm$ 0.16
30 nmol/L	1.1 $\pm$ 0.03*	1.6 $\pm$ 0.08
40 nmol/L	1.1 $\pm$ 0.04*	1.6 $\pm$ 0.09
50 nmol/L	1.2 $\pm$ 0.09*	1.5 $\pm$ 0.07

Cells were incubated with sodium chlorate for 20 min, washed twice and allowed to migrate toward AT III (1 U/mL) or RANTES (10 ng/mL) for 60 min. Chemotaxis was performed in modified Boyden chambers. Data are expressed as chemotaxis index. The distance of migration toward medium was $92.2 \pm 5.6 \mu\text{m}$ ($n = 3$). Statistical analysis: Kruskal–Wallis followed by Mann–Whitney *U*-test; * $P < 0.05$ vs. control. RANTES, regulated upon activation normal T cell expressed and activated; AT III, anti-thrombin III.

Table 3. Effect of blocking of signalling enzymes on leukocyte migration toward AT III

Treatment	AT-induced (1 U/mL) chemotaxis; chemotaxis index (mean $\pm$ SEM)		
	Eosinophils	Lymphocytes	Neutrophils
Medium	1.51 $\pm$ 0.03	1.65 $\pm$ 0.09	1.56 $\pm$ 0.04
Rolipram	1.16 $\pm$ 0.02*	0.99 $\pm$ 0.1*	1.03 $\pm$ 0.04*
Wortmannin	1.12 $\pm$ 0.12*	0.86 $\pm$ 0.01*	1.39 $\pm$ 0.10
Staurosporine	1.13 $\pm$ 0.07*	1.61 $\pm$ 0.15	1.18 $\pm$ 0.05*
GFX	1.09 $\pm$ 0.05*	1.08 $\pm$ 0.04*	1.07 $\pm$ 0.05*
Trypstin	1.37 $\pm$ 0.06	1.15 $\pm$ 0.13*	1.46 $\pm$ 0.01

Cells were incubated with enzyme blockers for 30 min, washed twice and allowed to migrate toward AT III (1 U/mL). Chemotaxis was measured in modified Boyden chambers. Data are expressed as chemotaxis index. The distance of random migration was 97.2 ± 6.98 , 66.5 ± 3.5 and 49.0 ± 8.0 for eosinophils, lymphocytes and neutrophils, respectively. Statistical analysis: Kruskal–Wallis followed by Mann–Whitney *U*-test; * $P < 0.05$ vs. pre-treatment with medium; $n = 3$. AT III, anti-thrombin III; GFX, GF 109203X.

implicates syndecan-4 of eosinophils in cell migration. AT III, which ligates with syndecan-4 [17], induced migration and blocked the chemotaxis of eosinophils toward chemokines. This effect could be reversed by heparinase-I, chondroitinase, chlorate and anti-syndecan-4 antibodies. Expression of the syndecan-4 gene in eosinophils was confirmed by using PCR.

Besides inflammation, activation of the coagulation system plays an important role in the pathogenesis of allergic diseases [18, 19]. Studies suggested that excessive pro-coagulant activity and abnormal fibrin turnover play relevant roles in the pathogenesis of atopic dermatitis and asthma bronchiale. Interstitial and intra-alveolar fibrin, its degradation products and local thrombin generation may potentiate the inflammatory response by their effects on chemotaxis, vascular permeability and immunomodulation [20]. AT III has anti-inflammatory properties, which are at least in part due to the promotion of endothelial prostacyclin release [21]. In an *in vivo* endotoxaemia model, a reduction of leucocyte adhesion by AT III is mediated by prostaglandin [22]. Others [23] reported that lung allograft rejection in animal studies is

affected by AT III. Additionally, they identified AT III as being inhibitory in T cell proliferation by concanavalin A. So besides established prostacyclin-mediated effects, AT III was also demonstrated to affect T cell proliferation [23].

For further studying direct effects of AT III on leucocytes, we used an experimental *in vitro* system that employed isolated eosinophils. Such an experimental set-up excludes to a large extent indirect effects, e.g. those mediated via thrombin antagonism by the serpin-related action of AT III. Also in this study, AT III failed to interact with thrombin in eosinophils. Ligation of HSPG with AT III not only altered endothelial cell function but also peripheral blood neutrophil [10], monocyte and lymphocyte [11] functions. It was previously reported that effects of IL-5 on IL-5-dependent cell lines are modulated by HSPGs [24]. HSPGs are important participants in cell surface signalling and critical in controlling cell behaviour. They modulate inflammatory cell maturation and activation, leucocyte rolling, adhesion to endothelium as well as extravasation and chemotaxis [5]. In the present study we observed direct chemotactic effects of AT III on eosinophils. Furthermore, AT III was able to decrease chemokine-induced eosinophil migration. Chemokines bind to G-protein-coupled seven transmembrane receptors, and for most chemokines distinct receptors have been identified [25]. Nevertheless, there appears to be quite some redundancy in chemokines and their appropriate receptors, on the one hand, and their action on target cells, on the other [26]. Thus, besides its specific receptor binding, chemokines bind to GAGs on leucocytes and GAGs are postulated as being modified in their properties and interactions [27]. Further study is required to explore an involvement of chemokine receptors in the mechanisms of action of AT III.

In order to verify specific binding of AT III to HSPG, we employed the specificity of heparinase I to highly sulphated polysaccharide chains containing linkages to 2-*O*-sulphated α -L-idopyranosyluronic acid residues [28]. Heparinase I has recently been described to degrade the synthetic heparin pentasaccharide to a disaccharide and trisaccharide product [29]. Pre-treatment of eosinophils with heparinase I abolished the migratory response of the leucocytes to direct exposure to AT III as well as their deactivation of chemokine-induced migration. In contrast, chemokine-induced chemotaxis or deactivation induced by a combination of attractants other than AT III was not affected by heparinase I. From this it can be concluded that the pentasaccharide sequence of HSPG of eosinophils, which is cleaved by heparinase I, is important for mediating the effects of AT III on the cells. Heparan sulphate chains abound on syndecans and glypicans and can bind a diverse repertoire of proteins. Thereby, HSPGs can immobilize the ligands, increase its local concentration, change its conformation, present it to a signalling receptor and enhance the formation of receptor–ligand signalling complexes [8]. Conceivably, modifications of these HS chains could influence any of the functions mentioned above. By cleaving this specific site known to bind and respond to AT III, chemotactic effects as well as deactivation in response to AT III were abolished.

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

sulphate and chondroitin sulphate, exemplify hybrid proteoglycans. In contrast, the glypicans appear to contain only heparan sulphate chains [30, 31]. To examine whether chondroitin sulphate proteoglycans act as putative cell receptors for AT III, eosinophils were treated with chondroitinase before testing cell migration. Data demonstrating that AT III's effects on cell migration are sensitive to both heparinase I and chondroitinase suggest that syndecans rather than glypicans mediate direct cellular actions of AT III.

To further substantiate the role of GAGs in the response, eosinophils were purified and pre-incubated with medium containing the GAG sulphation inhibitor sodium chlorate [32]. Such pre-treatment abolished responsiveness of the cells to AT III but not of RANTES, thus further supporting a role of GAGs in controlling leucocyte functions. These results are confirmed by the work of Culley et al. [33], who also demonstrated that proteoglycans are potent modulators in the biological response of eosinophils. Moreover, they revealed that treatment of eosinophils with proteoglycanases and chlorate does not abolish chemotaxis to CCL11, supporting the fact that two different mechanisms exist in migration, i.e. proteoglycan-dependent and proteoglycan-independent mechanisms.

We ligated the heparin-binding sequence on AT III by the synthetic pentasaccharide SR90107A before testing the effects in chemotaxis experiments. Previously, binding of the pentasaccharide to AT was shown to induce a conformational change that results in an accelerated inhibition of blood coagulation factor Xa by AT III [34], but this conformational change is insufficient to induce inhibition of thrombin by AT III [35]. Pre-incubation of AT III with the pentasaccharide led to a complete disappearance of its chemotactic effect, thus highlighting the essential role of the pentasaccharide-binding sequence on AT III. The molar ratio of effective concentrations of AT III and pentasaccharide in the experiments described suggests that SR90107A is able to neutralize functionally an excess of AT III. This may be explained by dissociation of the pentasaccharide after induction of a conformational change in AT III that prevents it from being active on HSPG. Data indicate that AT III affects eosinophil locomotion via its pentasaccharide-binding sequence through cell surface HSPG.

Syndecan-4 has a role in focal adhesion formation, and evidence also suggests that PKC activity is required both for focal adhesion formation and syndecan-4 insertion into forming adhesions [36, 37]. Its signalling properties have been described [38–40]. Syndecan-4 exists on B lineage lymphocytes [41], a variety of mature macrophage-like cells [42], neutrophils [17], lymphocytes and monocytes [11]. Using RT-PCR analyses of syndecan-4 of eosinophils, we confirmed syndecan-4 gene expression in eosinophils. The observation that AT III-affected migration of the cells was specifically inhibited by anti-syndecan-4 antibodies provides functional confirmation of the involvement of syndecan-4-typical signalling of AT III in eosinophils.

Syndecan-4 is alone among the family of cell surface HSPG in having a cationic motif in its cytoplasmatic V region that can bind PKC α and inositol phospholipids [43]. It has been shown that phosphatidylinositol 4,5-bisphosphate is essential for focal adhesion assembly, and further data suggest that one function may revolve around the capacity of syndecan-4

to signal through PKC α [44]. In our experiments the specific PKC inhibitor GFX and the selective PDE₄ inhibitor rolipram attenuated AT III-induced chemotaxis in eosinophils, neutrophils and lymphocytes. PDE₄ dependence of lymphocyte chemotaxis toward IL-8 has previously been demonstrated [45] and may thus not be specific for AT III. Hence, different cell types show distinct patterns of signalling upon HSPG ligation with AT III. Alternatively, different GAGs on leucocyte subpopulations may be involved in AT III-induced signalling, syndecan-4 being only one of them. However, further elucidation of the mechanisms involved is necessary in order to provide a better understanding of the signalling events elicited in AT III-induced alteration of migration in different leucocyte populations.

Our results provide strong evidence for the interaction of AT III with heparan sulphate and chondroitin sulphate chains attached to proteoglycans on the surface of eosinophils leading to a previously unknown specific action of AT III on immune cell populations. Biochemical and functional tests identify syndecan-4 as a putative functional receptor for AT III, which in this context acts via its heparin-binding site. These observations may have some relevance in the cell biology of leucocyte-matrix interactions in allergic reactions.

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