Cell-surface heparan sulfate proteoglycan–mediated regulation of human neutrophil migration by the serpin antithrombin III

Stefan Dunzendorfer, Nicole Kaneider, Andrea Rabensteiner, Christian Meierhofer, Christina Reinisch, Jürgen Römisch, and Christian J. Wiedermann

The serpin antithrombin III (AT III) is reported to have hemostasis-regulating and anti-inflammatory properties. To determine its ability to influence thrombin-independent leukocyte responses, the direct effects of the AT III concentrate Kybernin P and a monoclonal antibody-purified AT III on neutrophil migration were studied. Chemotactic activity of human neutrophils isolated from the blood of healthy donors was determined in modified Boyden microchemotaxis chambers, and binding studies were performed according to standard experimental protocols. Preincubation in vitro of neutrophils with Kybernin P or immune-adsorbed AT III significantly deactivated migration toward FMLP, or interleukin-8 (IL-8), in a concentration-dependent manner. In the absence of additional attractants, neutrophils exhibited a migratory response toward gradients of AT III preparations. True chemotaxis was confirmed in checkerboard assays. Analyses revealed that the AT III heparin-binding site interacts with neutrophil membrane–associated heparan sulfate proteoglycan receptors. Mechanisms of intracellular signaling differed; the deactivation of IL-8–induced chemotaxis resulted from tyrphostin-sensitive interactions of AT III-signaling with the IL-8 signal transduction pathway, whereas AT III–induced chemotaxis involved protein kinase C and phosphodiesterases. Signaling similarities between AT III and the proteoglycan syndecan-4 may suggest the binding of AT III to this novel type of membrane receptor. Under physiological conditions, AT III may prevent neutrophils from premature activation. Moreover, the systemic administration of AT III concentrate could have beneficial effects in combating systemic inflammation. (Blood. 2001;97:1079-1085)

Introduction

Activation of neutrophils plays a crucial role in the development of tissue injury and is a hallmark of every inflammatory process. Several endogenous mediators have been described to prevent the organism from conditions of uncontrolled neutrophil migration and enzyme release. Disseminated intravascular coagulation is an early complication in patients with sepsis and may be a precipitating factor for complications such as acute respiratory distress syndrome. In this context, oxidants, cytokines, and proteinases play a major role in perpetuating inflammation. Not only cytokine-mediated activation of the coagulation system but also cytokine-induced leukocyte activation is an important step in the onset of the septic response and contributes to septic shock. In addition to exogonous bacterial-derived formyl-Met-Leu-Phe (fMLP), the chemokine interleukin-8 (IL-8) is one of the most potent endogenous proinflammatory mediators, and its role in leukocyte activation of sepsis is well established.

Besides preventing hemorrhagic complications, antithrombin III (AT III) exerts anti-inflammatory effects by inhibiting thrombin and other activated clotting proteases. It has been shown to be efficacious in several animal models of sepsis. The hypothesis that AT III can reduce mortality in patients suffering from severe sepsis is being tested in an ongoing large-scale phase III clinical trial. Additionally, thrombin-independent effects exerted more directly on leukocytes and endothelial cells have also been reported. Previously, the direct action of serpins on leukocyte migration, which is independent of serpin-enzyme complex formation, has been proposed for heparin cofactor II, which is an AT III analogue. Recently, antiangiogenic properties of conformation-modified AT III have been described.

Mechanisms by which AT III exerts its protective effect in systemic inflammation are thus far only partly understood. Because not only the anti-DIC disseminated intravascular coagulation activity of AT III in early sepsis but also thrombin-independent anti-inflammatory effects may involve neutrophils and could, therefore, be responsible for an increased survival in sepsis models, we investigated the impact of this serpin on human neutrophil function. Given that the effects of AT III on neutrophils occur at normal plasma concentrations below 1 U AT III, our findings could be of physiological relevance.

Materials and methods

Neutrophil isolation

Neutrophils were obtained from EDTA–anticoagulated peripheral blood of healthy donors after discontinuous density gradient centrifugation on Percoll by dextran sedimentation and centrifugation through a layer of Ficoll-Hypaque, followed by hypotonic lysis of contaminating erythrocytes using sodium chloride solution. Cell preparations yielded more than 95% neutrophils (by morphology in Giemsa stains) and more than 99% viability (by trypan dye exclusion). Experiments were performed in RPMI 1640
Chemical) Then the cells were washed before migration. Cells were
sp), tyrphostin, iso-butyl-methylxanthine (IBMX), or wortmannin for 30
Streptomyces blockers bisindolylmaleimide I GF 109203X (GFX; Bo ¨hringer Ingelheim
Migration of neutrophils into cellulose nitrate to gradients of soluble
Neutrophil chemotaxis
separated by the distinct cleavage with elastase.
products if run under nonreducing conditions. As expected, after reduction
cant difference in the starting material with respect to potential cleavage
detectable in the chromogenic test system. SDS-PAGE showed no signifi-
taxis assay. Neither elastase activity nor elastatinal inhibitory activity was
described above. Incubation with elastase led to a complete destruction of
substrate S 2484 (Chromogenix AB). A T III activity was determined as
inhibitor. Residual elastase activity was measured using the chromogenic
to gel filtration (SEC-HPLC) to remove the excess of the LMW-elastase
terminated with the addition of 35
m
CO2 ). In some experiments, cells were treated with the intracellular enzyme
the lower chambers for 35 minutes at 37°C in a humidified atmosphere (5%
were washed twice in phosphate-buffered saline (PBS), neutrophils mi-
toward IL-8 served as a positive control. For deactivation, neutrophils were
incubated with human monoclonal antibodies (mAb) to sialyl Lewis(x),
IL-8 receptors (CXCR1 and CXCR2) (Biomeda, Foster City, CA), or CD45
(Bra55) (Sigma Chemical) for 2 hours. To exclude thrombin contaminations
or the effects of neutrophil-derived elastase, hirudin (Sigma Chemi-
cal) or a neutrophil elastase inhibitor (Zeneica Pharmaceuticals, Södertälje,
Sweden) was present during cell migration in 2 sets of experiments. The
assay was performed as recently described.19

Checkerboard analyses
To ensure that the effect observed was true chemotaxis, checkerboard
analyses were performed. Neutrophils were resuspended in RPMI 1640–
0.5% BSA containing various concentrations of AT III or Kybernin P just
before they were transferred to the upper chamber. The same concentrations
of the respective substances remained beneath the filter of the Boyden
chamber; thus, distinct concentration gradients could be formed. Data are
expressed as chemotaxis index within a matrix.

Receptor-binding analyses
Human neutrophils (107 cells/mL) were incubated with sodium iodide I
125–IL-8 (2000 Ci/mM) at concentrations of 10 fM/L plus minus an
excess of unlabeled IL-8, or AT III at various concentrations, in a total
volume of 400 mL PBS–0.5% BSA, for 90 minutes at 4°C. After incubation,
cells were washed twice with PBS–0.5% BSA containing 0.5 mol/L saline and
centrifuged at 200g for 10 minutes. The bottom of the tube containing
the neutrophil pellet was counted in a Beckman gamma counter. Results are
expressed as counts per minute (cpm). Specific binding is defined as total
binding minus nonspecific binding, which is the residual cpm bound in the
presence of 100-fold excess unlabeled IL-8.21 IL-8 receptor internalization
deactivation) was measured by the preincubation of cells with IL-8, IMLP,
or AT III for 5 minutes according to previous time-course experiments.
For homologous receptor desensitization experiments, IL-8 (0.1-10 nM/L)
was used, and for heterologous desensitization, IMLP (10 nM/L–1 mM/L)
or AT III (0.01-1 U/mL) was used. Thereafter, total 125I–IL-8 binding
was quantified.

Statistical methods
Data are expressed as mean ± standard error of the mean (SEM). The
chemotaxis index is the ratio between the distances of directed
and undirected migration of neutrophils into the nitrocellulose filters. Means
were compared by Mann Whitney test and Kruskal-Wallis analysis of
variance. P < .05 was considered significant.

Results
Induction of directed migration and deactivation of chemotaxis
by AT III or Kybernin P
Preincubation of neutrophils with either immune-adsorbed AT III
or Kybernin P at various concentrations decreased the migration of
cells to IMLP and IL-8. Significant inhibition occurred at concentra-
tions higher than 0.1 mU (Figure 1A-B). The flow-through fraction
did not produce comparable results (not shown). Pretreatment with
IL-8, which resulted in homologous deactivation, served as a
positive control. The AT III–induced effect was strictly time
dependent, with significant values appearing after 3 minutes of
preincubation and reaching a plateau after 10 minutes (Figure 1C).
AT III or Kybernin P put beneath the chemotaxis-filter was able to
induce directed cell migration. Maximal stimulation was observed
at doses between 10 mU and 1 U; further increasing the concentra-
tion up to 5 U diminished the dose-response (Figure 1D). In
checkerboard analyses the migratory response was confirmed as
true chemotaxis (Table 1).
Neutrophils. However, the mAbs themselves slightly induced cell migration; antibodies significantly diminished A T III–induced migration of neutrophils. Except the control mAb, all mAbs reduced migration (Table 2). Means of chemotaxis indices were compared by Mann-Whitney test. Deactivation of neutrophil chemotaxis to IL-8 (C). Directed migration of neutrophils to IL-8, CI 1.266, 90% CI 1.107–1.391; p < .01. n = 13 (A,B); n = 5 (C); n = 9 (D).

**Effects of AT III** or TAT complexes on neutrophil migration**

In contrast to TAT (10 nU-1 U), which was completely ineffective, AT III (10 nU-1 U) mimicked the effects of AT III regarding the deactivation of IL-8–induced chemotaxis and the induction of directed migration at comparable effective dose ranges (Figure 2). The thrombin inhibitor hirudin, at various concentrations, also failed to affect AT III–induced chemotaxis; moreover, a neutrophil elastase inhibitor lacked such an effect when both substances were present during the migration of cells (Table 2).

**Inhibition of AT III–induced chemotaxis by anti–IL-8 receptor antibodies**

Neutrophils were incubated for 2 hours with blocking antibodies to CXCR1, CXCR2, or both or with an anti–IL-1 receptor control mAb. Migration to fMLP (10 nM) was not disturbed by any of the mAbs used. Except the control mAb, all mAbs reduced migration to IL-8 (1 nM). AT III–induced chemotaxis was insensitive to the pretreatment of cells with control mAb or anti–CXCR2 mAb. In contrast, anti-CXCR1 or a combination of both anti–IL-8 receptor antibodies significantly diminished AT III–induced migration of neutrophils. However, the mAbs themselves slightly induced cell migration (Figure 3).

**Table 1. Effect of concentration gradients of Kybernin P or antithrombin III on neutrophil migration**

<table>
<thead>
<tr>
<th>Gradient</th>
<th>Upper chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^{-6}</td>
</tr>
<tr>
<td>Kybernin P (u)</td>
<td></td>
</tr>
<tr>
<td>Lower chamber</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>1.009 ± 0.009</td>
</tr>
<tr>
<td>AT III (u)</td>
<td></td>
</tr>
<tr>
<td>Lower chamber</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>1.009 ± 0.009</td>
</tr>
</tbody>
</table>

Statistical analyses: Mann-Whitney test after Kruskal-Wallis analysis of variance (P < .001); n = 5.

*AT III indicates antithrombin III.*

**P < .01 vs. highest concentration of test substance exclusively in the lower chamber. Only those groups within the matrix being sufficient to distinguish between chemokinesis and chemotaxis were compared statistically by Mann-Whitney test.**

**IL-8 receptor binding studies**

Concomitant incubation of neutrophils with radioactive labeled and various concentrations of “cold” IL-8, but not with AT III, displaced125I–IL-8 (Figure 4). To investigate receptor internalization, we preincubated cells with IL-8 (0.1-10 nM), fMLP (0.01-1 nM), or AT III (0.01-1 U) before total binding was quantified. IL-8 or fMLP pretreatment dose-dependently down-regulated IL-8 receptor expression, whereas AT III treatment did not (Figure 4).

**Effects of blocking sialyl Lewis(x)**

Sialyl Lewis(x) (CD15) mAb binding to the neutrophil surface did not affect random migration. Incubation of untreated cells with AT III (1 U) for 20 minutes diminished chemotaxis to IL-8 (1 nM), as described above (IL-8, CI 1.997, ±.17; AT III + IL-8, CI 1.573, ±.06), but the blockade of CD15 could not abolish the AT III effect.

**Figure 1. Deactivation of chemotaxis and directed migration induced by AT III or Kybernin P.** Neutrophils were pretreated for 20 minutes with Kybernin P or AT III before migration to fMLP or IL-8 was tested. Homologous deactivation by IL-8 served as a positive control (A,B). Time-course of pretreatment for AT III– or IL-8–induced deactivation of neutrophil chemotaxis to IL-8 (C). Directed migration of neutrophils to Kybernin P or AT III (D). Migration time into nitrocellulose filters was 30 minutes. After fixing and staining of filters, migration depth was quantified microscopically. The chemotaxis index is the ratio between the distances of directed and undirected migration of neutrophils.

**Figure 2. Effects of AT III ED or TAT complex on neutrophil migration.** Untreated neutrophils migrated to various concentrations of AT III (u) or TAT complexes (●). AT III and IL-8 served as positive controls (left). For deactivation experiments, cells were pretreated with either AT III (u) or TAT complexes for 20 minutes before chemotaxis to IL-8 was run. Preincubation with AT III was the positive control (right). Cells migrated for 30 minutes into nitrocellulose filters, and, after fixing and staining of filters, migration depth was quantified microscopically. The chemotaxis index is the ratio between the distances of directed and undirected migration of neutrophils. Means of chemotaxis indices were compared by Mann-Whitney test after Kruskal-Wallis analysis of variance (P < .001); **P < .01. n = 5.
Table 2. Failure of hirudin or neutrophil elastase inhibitor to affect antithrombin III–induced chemotaxis or deactivation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neutrophil migration versus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium IL-8 [1 nM] AT III [1 U]</td>
</tr>
<tr>
<td>Medium</td>
<td>1.000 ± 0.00 2.147 ± 0.18 —</td>
</tr>
<tr>
<td>AT III [1 U]</td>
<td>1.045 ± 0.07 1.476 ± 0.06 —</td>
</tr>
<tr>
<td>+ Ei [0.1 μM/L]</td>
<td>1.105 ± 0.03 1.537 ± 0.12ns —</td>
</tr>
<tr>
<td>+ Ei [1 μM/L]</td>
<td>0.978 ± 0.05 1.301 ± 0.14ns —</td>
</tr>
<tr>
<td>+ Ei [10 μM/L]</td>
<td>1.006 ± 0.03 1.509 ± 0.15ns —</td>
</tr>
<tr>
<td>+ Ei [100 μM/L]</td>
<td>1.104 ± 0.08 1.313 ± 0.13ns —</td>
</tr>
<tr>
<td>IL-8 [1 nM]</td>
<td>1.201 ± 0.09 1.132 ± 0.11 —</td>
</tr>
<tr>
<td>Medium</td>
<td>1.000 ± 0.00 — 1.584 ± 0.07</td>
</tr>
<tr>
<td>Hirudin [0.01 mg/mL]</td>
<td>1.159 ± 0.03 — 1.484 ± 0.11ns</td>
</tr>
<tr>
<td>Hirudin [0.1 mg/mL]</td>
<td>1.150 ± 0.09 — 1.566 ± 0.10ns</td>
</tr>
<tr>
<td>Hirudin [1 mg/mL]</td>
<td>1.150 ± 0.08 — 1.428 ± 0.09ns</td>
</tr>
<tr>
<td>Hirudin [5 mg/mL]</td>
<td>1.037 ± 0.04 — 1.481 ± 0.08ns</td>
</tr>
</tbody>
</table>

IL-8 indicates interleukin-8; AT III, antithrombin III; ns, not significant.

(anti-CD15 mAb + AT III + IL-8, CI 1.349 ± .10; not significant compared with AT III + IL-8).

Influence of leukocyte common antigen ligation

Ligation of leukocyte common antigen (CD45) by Bra55 significantly diminished the migration of neutrophils to fMLP (10 nM), IL-8 (1 nM), or AT III (1 U). These effects were tyrphostin sensitive only when IL-8 or AT III was used for chemotaxis, whereas CD45-dependent deactivation of fMLP-induced chemotaxis was tyrphostin insensitive. The same was true of homologous deactivation by AT III of AT III–induced chemotaxis. In contrast, AT III deactivating properties of directed migration triggered by IL-8 and fMLP could be reversed by tyrsoine kinase inhibition (Table 3).

Blockade of LDL receptor–related protein by RAP

IL-8–induced chemotaxis was blocked by AT III (1 U) and by RAP (5 μg/mL). When neutrophils were exposed concomitantly to AT III and RAP before chemotaxis was tested, RAP failed to affect AT III chemotaxis to IL-8 was not restored. However, RAP (0.001–10 μg/mL) was capable of diminishing directed migration to AT III (Figure 5) and itself induced the chemotaxis of neutrophils (Figure 5, inset), suggesting cross-deactivation between AT III and RAP.

AT III effects after cleavage of pentasaccharide by heparinase

Neutrophil surface heparan sulfate proteoglycan pentasaccharide was cleaved by the treatment of cells with heparinase (50 mU/mL). This neither affected random migration nor chemotaxis of neutrophils to fMLP (10 nM), AT III (1 U)–induced chemotaxis, however, was completely abolished. In addition, the deactivation properties of AT III disappeared and AT III was no longer able to diminish neutrophil chemotaxis to IL-8 (1 nM) (Figure 6).

Signaling of AT III–induced neutrophil chemotaxis

When neutrophils were pretreated with intracellular enzyme blockers, the IL-8–induced chemotactic response was inhibited by GFX

Table 3. Effects of CD45-ligation on antithrombin III–induced chemotaxis and deactivation

<table>
<thead>
<tr>
<th>Chemotaxis versus</th>
<th>Medium IL-8 [1 nM/L] fMLP [10 nM/L] AT III [1 U]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45 Bra55 [5 μg/mL]</td>
<td>2.080 ± 0.18 1.632 ± 0.20 1.374 ± 0.08</td>
</tr>
<tr>
<td>CD45 Bra55 + Tyrophostin</td>
<td>1.469 ± 0.10 1.299 ± 0.11 1.103 ± 0.08</td>
</tr>
<tr>
<td>AT III [1 U]</td>
<td>2.215 ± 0.12 1.367 ± 0.22ns 1.338 ± 0.04*</td>
</tr>
<tr>
<td>AT III + Tyrophostin</td>
<td>2.150 ± 0.07 1.119 ± 0.11 1.030 ± 0.04</td>
</tr>
<tr>
<td>CD45 Bra + AT III + Tyrophostin</td>
<td>1.904 ± 0.10* 1.343 ± 0.13* 1.194 ± 0.10 ns</td>
</tr>
</tbody>
</table>

Mann-Whitney test after Kruskal-Wallis analysis of variance (P < .01); n = 5. For abbreviations, see Table 2. *P < .01.
(500 nM), staurosporine (10 ng/mL), and IBMX (100 μM), whereas tyrophostin (10 ng/mL) and wortmannin (10 nM) lacked this effect. AT III (1 U)–induced neutrophil migration mimicked this signal transduction pattern. Random migration of cells remained unaffected by their pretreatment with enzyme blockers (Table 4).

![Figure 5. Impact of RAP on AT III effects in neutrophils. In deactivation experiments, cells were pretreated for 20 minutes with AT III, RAP, or both concomitantly. Thereafter, IL-8–induced chemotaxis was tested (left). Chemotaxis to AT III was investigated after the treatment of cells with RAP at various concentrations (right). In addition, untreated neutrophils were allowed to migrate to various concentrations of RAP (inset). Migration time to nitrocellulose filters was 30 minutes. After fixing and staining of filters, migration depth was quantified microscopically. The chemotaxis index is the ratio between the distances of directed and undirected migration of neutrophils. Means of chemotaxis indices were compared by Mann-Whitney test after Kruskal-Wallis analysis of variance (P < .01); n = 6.](Image)

![Figure 6. Impact of heparinase-induced cleavage on AT III effects in neutrophils. Heparinase-treated (50 minutes) or -untreated neutrophils migrated for 30 minutes to AT III at various concentrations. Chemotaxis induced by fMLP or IL-8 served as positive controls (left). In deactivation experiments, heparinase-treated or -untreated neutrophils were exposed to AT III for 20 minutes; thereafter, migration to medium or IL-8 was run (right). The chemotaxis index is the ratio between the distances of directed and undirected migration of neutrophils. Means of chemotaxis indices were compared by Mann-Whitney test after Kruskal-Wallis analysis of variance (P < .001). *P < .05; **P < .01. ns, not significant; n = 8.)](Image)

![Table 4. Antithrombin III–induced signaling mediating neutrophil chemotaxis](Image)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Chemotaxis versus</th>
<th>Medium</th>
<th>AT III [1 U]</th>
<th>IL-8 [1 nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>1.000 ± 0.00</td>
<td>1.491 ± 0.06</td>
<td>1.718 ± 0.16</td>
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</tr>
<tr>
<td>GFX [500 nM]</td>
<td>1.054 ± 0.09</td>
<td>1.192 ± 0.08*</td>
<td>1.387 ± 0.10*</td>
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<tr>
<td>Staurosporine [10 ng/mL]</td>
<td>0.980 ± 0.12</td>
<td>1.053 ± 0.10*</td>
<td>1.181 ± 0.16*</td>
<td></td>
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<tr>
<td>Tyrophostin [10 ng/mL]</td>
<td>1.105 ± 0.08</td>
<td>1.354 ± 0.04ns</td>
<td>1.757 ± 0.11ns</td>
<td></td>
</tr>
<tr>
<td>IBMX [100 μM]</td>
<td>1.113 ± 0.10</td>
<td>1.124 ± 0.07*</td>
<td>1.212 ± 0.04*</td>
<td></td>
</tr>
<tr>
<td>Wortmannin [10 nM]</td>
<td>0.965 ± 0.09</td>
<td>1.349 ± 0.07ns</td>
<td>1.971 ± 0.17ns</td>
<td></td>
</tr>
</tbody>
</table>

Mann-Whitney test after Kruskal-Wallis analysis of variance (P < .01); n = 6. For abbreviations, see Table 2.

*P < .05.

**Discussion**

Both AT III preparations, Kybernin P and the immune-adsorbed AT III of the concentrate, deactivated IL-8 and fMLP-induced neutrophil chemotaxis. A monoclonal antibody–column flow-through fraction had no comparable effect, thus excluding the effects of unidentified contaminations. Such inhibition of chemokine-triggered chemotaxis of neutrophils could also have occurred in the study by Ostrovsky et al.,14 who showed that pretreatment and posttreatment in a feline model of mesentery ischemia–reperfusion with AT III reduced neutrophil rolling and adhesion to baseline levels. They explained the observed in vivo effect exclusively in terms of the thrombin-inhibiting properties of AT III.14 In our study, neutrophil chemotaxis to fMLP and IL-8 was diminished at physiological concentrations of purified AT III in the absence of thrombin because cells were isolated and experiments in the presence of the thrombin antagonist hirudin did not yield different results. In time-course experiments the AT III–induced process of chemotaxis deactivation occurred rapidly and the chemotactic effects of AT III and Kybernin P on neutrophils were confirmed in checkerboard analyses; this response pattern and the bell-shaped dose-response curve of neutrophil chemotaxis directly evoked by AT III clearly indicate a receptor-mediated process. Because interactions between the AT III molecule and a supposed receptor on neutrophils require binding sites on both, we further investigated this issue.

**The molecule**

AT III is a typical serpin with 432 residues, and it contains 9 helices and 3 β-sheets.22,23 The reactive site (thrombin binding) is located at Arg393-Ser394 in the COOH-terminal loop structure, whereas the heparin-binding site is part of the NH2-terminal region.24 Because TAT complexes completely failed to induce neutrophil migration and lacked deactivating IL-8–induced chemotaxis, one might assume that it is the thrombin-binding site that is mainly involved. This concept had to be rejected when elastase-digested AT III, in which absolutely no thrombin inhibitory action was left, completely mimicked the effects of the full functional AT III molecule, suggesting that conformational changes occurring in the course of complex formation23,25 are responsible for the failure of TAT complexes to influence neutrophil function.

We could also demonstrate that neutrophil elastase, which plays an important role in the pathogenesis of acute inflammation,26 is not the driving force that makes AT III chemotactic given that the presence of an elastase inhibitor had no effect on neutrophil migration.

It may be the responsibility of the heparin-binding site to interact with cell surface receptors. The property of heparin binding...
is almost wholly owing to a small pentasaccharide fragment that binds to AT III, and the occurrence of a specific conformational change, together with the structural specificity of pentasaccharide, implies a well-defined binding site on the molecule.27

The receptor
In searching for a receptor that mediates both the deactivation of chemotaxis and the stimulation of directed migration, several experimental approaches were applied. Because of the initial results on AT III–induced deactivation of IL-8 chemotaxis, it was obvious that we should look for interactions with the 2 CXC receptors.28 Surprisingly, we found inhibitory action of AT III–induced neutrophil chemotaxis by monoclonal antibodies to the IL-8 receptors, with CXCR1 mAb having a higher potency. Given that the antibodies themselves slightly induced cell migration, this effect might have resulted from weak stimulation of the IL-8 pathway and, therefore, might have mimicked the phenomenon described above. In subsequent binding assays, neither specific binding of AT III to IL-8 receptors nor AT III–induced receptor internalization29 could be shown.

Leukocyte common antigen (CD45) is a transmembrane protein tyrosine phosphatase expressed on all nucleated hematopoietic cells30 and leukocytes, the ligation of which has been reported to modulate IL-8 responsiveness.31 Pretreatment of neutrophils with the anti-COX-2 mAb BrA11 reduces cells surface expression of CXCR1 and CXCR2.32 This effect is completely tyrphostin reversible.32 We used BrA55, another anti-COX-2 mAb that deactivates neutrophil chemotaxis without affecting receptor density,32 and found that the inhibition of AT III–induced directed migration by BrA55 was reversed by tyrphostin. Furthermore, the addition of tyrphostin abrogated AT III–induced deactivation of IL-8–triggered chemotaxis and thereby restored the migration of neutrophils to IL-8. In contrast, homologous deactivation of AT III chemotaxis was insensitive to the tyrosine kinase inhibitor. Insensitivity to tyrphostin was also observed for AT III–induced chemotaxis (see below). Thus, our results demonstrate that the so-called deactivating properties of AT III are related to the activation of CD45 PTPase-dependent, discrete signaling pathways that also interfere with IL-8 signaling, whereas its chemotactic action uses a distinct signal transduction that does not involve tyrosine kinases. Because an antibody to sialic Lewis(x) did not disturb the interactions of AT III with neutrophils, any involvement of CD15 can be excluded, and an antibody to sialyl Lewis(x) did not disturb the interactions of AT III with neutrophils, any involvement of CD15 can be excluded. Moreover, the results from signal transduction experiments may suggest involvement of syndecan-4. In contrast to other proteoglycans related to this family, the core protein of syndecan-4 can directly bind the catalytic domain of protein kinase C, and it activates this enzyme in the presence or absence of phospholipid mediators.34,45 This is consistent with the susceptibility of AT III–evoked chemotaxis to protein kinase C inhibitors (bisindolylmaleimide I, staurosporine) and a phosphodiesterase inhibitor (IBMX).

Conclusion
To summarize, our results revealed that AT III affects neutrophil functions by binding to heparan sulfate proteoglycans. Distinct signal transduction pathways are involved in the deactivation of IL-8–induced chemotaxis and in AT III–stimulated directed migration. As do AT III and related–related effects, syndecan–induced signaling can cross-react with IL–8–activated signal transduction, which, in turn, leads to deactivation.46 Neutrophil chemotaxis to AT III seems to be directly protein kinase C dependent. The overall finding is consistent with the interferon–related effect of AT III administration in vivo. During circulation neutrophils are continuously exposed to AT III in plasma; therefore, this serpin may also protect neutrophils from premature activation under physiological conditions. Surprisingly, there are no reports of abnormalities in neutrophil function or recurrent infection in patients with congenital AT III deficiency. Additional studies are needed for clarifying the physiological role of AT III in leukocyte functions.

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