Antithrombin III Inhibits Thrombin-Induced Proliferation in Human Arterial Smooth Muscle Cells

Ulf Hedin, Siw Frebelius, Javier Sanchez, Maciej Dryjski, Jesper Swedenborg

Abstract Thrombin has attracted increasing attention as a possible mitogen for vascular smooth muscle cells in lesion development both after vascular injury and in atherogenesis. In this study, the ability of antithrombin III to inhibit α-thrombin-induced DNA synthesis and cell proliferation in human arterial smooth muscle cells was analyzed. We demonstrate a concentration-dependent inhibition of DNA synthesis and cell proliferation by α-thrombin. This effect was abolished when complex formation with antithrombin III was allowed before thrombin was added to the cell cultures. Addition of α-thrombin and antithrombin III simultaneously at the beginning of the incubation period also resulted in an inhibition of thrombin-induced DNA synthesis, but to a lower degree. The inhibitory activity of antithrombin III was enhanced in the presence of heparin, which on its own had no inhibitory effect on thrombin-induced DNA synthesis. In contrast, the mitogenic activity of α-thrombin could be inhibited by heparin in the presence of low concentrations of serum. This inhibition was dependent on the presence of antithrombin III in serum, since heparin lacked effect if antithrombin III was depleted from serum by immunoaffinity chromatography. Analysis of the enzymatic activity of thrombin showed that the influence on catalytic activity of thrombin corresponded to the mitogenic activity of thrombin in the presence of heparin, antithrombin III, and serum. The results suggest that the mitogenic activity of thrombin is regulated by antithrombin III. Therefore, antithrombin III may serve dual functions by inhibiting thrombin in the coagulation cascade and by neutralizing its growth-promoting effects on vascular smooth muscle cells. (Arterioscler Thromb. 1994;14:254-260.)

Key Words • thrombin • mitogens • smooth muscle cells • antithrombin III • antiproliferative effects

The proliferation of arterial smooth muscle cells (SMCs) is a key event in the development of arterial lesions after vascular injury and in atherogenesis.1-3 The growth-regulatory mechanisms of SMCs have been postulated to depend on growth factors in the vessel wall acting by autocrine or paracrine mechanisms as well as by release of blood-borne mitogens.1,4 Mural thrombosis occurs on the vessel wall after vascular injury and may be enhanced after injury of atherosclerotic lesions.5-8 Thrombin plays a crucial role at several levels in the cascade reaction leading to thrombus formation,9 but it may also regulate many postthrombotic events.10 Clot-bound thrombin is gradually released into the extracellular matrix of the vascular wall,11 where it may influence endothelial and leukocyte function12 and participate in the growth regulation of SMCs.13-16

The role of thrombin as a possible mitogen for vascular cells has gained further support by the identification of a cellular thrombin receptor17,18 and by the recent detection of mRNA for this receptor in human atherosclerotic plaques.19 In addition, specific thrombin inhibitors, such as hirudin, reduce the proliferative response of SMCs in the arterial wall after vascular injury in animal models.20 The mechanisms by which thrombin induces proliferation of vascular SMCs are not entirely established. The cellular response elicited by binding of thrombin to its receptor includes G protein-mediated kinase activation15,16 and subsequent induction of platelet-derived growth factor A-chain (PDGF A) gene expression in SMCs15,21 as well as other growth-related genes.22,23

The main physiological inhibitor of thrombin coagulant activity is antithrombin III (AT III), which forms an inactive complex with thrombin. Thrombin/antithrombin III (TAT) complex formation is rapidly catalyzed by cell surface heparan sulfate proteoglycans or by intravenously administered heparin.24,25 Increased thrombin coagulant activity occurs after endothelial denudation by balloon catheterization,26 which can be neutralized by AT III accumulated on the vessel surface.27

Since AT III is the main inhibitor of the procoagulant properties of thrombin, we investigated the effects of AT III on the mitogenic response of thrombin in human arterial SMCs. The findings show that complex formation of thrombin with AT III efficiently abolishes its mitogenic effects on SMCs. In addition, the inhibitory action of heparin on thrombin-induced mitogenesis in SMCs was found to be largely dependent on AT III. Taken together, our results suggest that AT III acts as a physiological inhibitor of the mitogenic activities of fluid-phase thrombin.

Methods

Cell Culture

Human SMCs were isolated from macroscopically healthy iliac arteries obtained from cadaver donors at organ transplantation. The adventitia was dissected off and the intima removed by scraping with a scalpel. The medial layer was

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thereafter minced in pieces, and explant cultures were set up in 12-well plates in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Paisley, Scotland), 50 μg/mL L-asparaginase, 50 μg/mL streptomycin, and 20% fetal calf serum (FCS; GIBCO) and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. At confluency, the cells were detached by treatment with 0.1% trypsin (GIBCO) and 0.02% EDTA in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS; pH 7.4; GIBCO) and resuspended in 75-cm² flasks. The cultures were split by trypsinization at a ratio of 1:3 and used for experimental procedures between the fourth and the eighth passages.

**Immunofluorescence Microscopy**

The cells were characterized by indirect immunofluorescence microscopy using a monoclonal antibody against smooth muscle-specific α-actin (Sigma, St Louis, Mo), a monoclonal antibody against a human fibroblast cell surface antigen (Sigma) and a monoclonal antibody against von Willebrand factor (Dakopatts, Glostrup, Denmark). SMC cultures were set up on glass coverslips and grown in DMEM/20% FCS. The cells were fixed in 4% formaldehyde in PBS for 10 minutes and washed twice with PBS. The coverslips were then either permeabilized in PBS/0.5% Triton X-100 for 3 minutes or freshly incubated with primary antibodies followed by incubation with fluorescein-conjugated goat anti-mouse IgG. Primary and secondary antibodies were diluted in PBS with 1% bovine serum albumin (BSA), and the coverslips were incubated for 2 hours at 37°C. After rinsing in PBS, the coverslips were mounted in 90% glyceral with 0.1% paraffin-nylendiamine. The specimens were studied in a Nikon Labophot microscope with epifluorescence optics, and photographs were taken on Kodak Tri-X-Pan film.

**Assays of DNA Synthesis and Cell Proliferation**

Confluent SMC cultures were trypsinized and seeded in 24-well multidishes (5000 cells/cm²; 2 cm²). To measure DNA synthesis, the cells were grown to subconfluency in DMEM/20% FCS, and growth was arrested in DMEM/0.1% BSA for 48 hours. They were then growth-stimulated with human α-thrombin (thrombin; American Diagnostica, Greenwich, Conn), recombiant PDGF BB homodimer (PDGF B; a gift from Carl-Henrik Heldin, Ludwig Institute, Uppsala, Sweden), FCS, or newborn calf serum (NCS; GIBCO) diluted in DMEM/0.1% BSA with 1 μCi/mL [³H]thymidine (5 Ci/mmol; Amershams International, Amersham, UK). In some experiments, recombiant hirudin (r-hirudin; Pentapharm AG, Basel, Switzerland), AT III (a gift from L. Javelin, Kabi Pharmacia, Stockholm, Sweden), or heparin (porcine mucosal; 5000 U/mL; Kabi Pharmacia) was mixed with thrombin and incubated for 10 minutes before the solutions were added to the cell cultures. The cultures were rinsed at varying time intervals in PBS, and macromolecular material was precipitated with cold 5% trichloroacetic acid (TCA). After two rinses in TCA, the cells were dissolved in 0.1 mol/L KOH. Aliquots of the lysates (0.2 mL) were mixed with Ecoscint A (National Diagnostics, Manville, NJ), and radioactivity was determined in a liquid scintillation spectrometer (Packard Instrument Co, Meriden, Conn). Parallel wells were trypsinized and the cells counted in an electronic cell counter.

To follow the proliferative rate in the cultures, serum-starved cells were exposed to experimental medium, detached by trypsinization at the indicated intervals, and counted in an electronic cell counter. For prolonged experiments, the medium was changed every second day.

**Preparation of Antithrombin-Free Serum**

AT III was removed from human serum by immunoaffinity chromatography. Cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) was coupled with rabbit antihuman AT III (IgG fraction; Dakopatts) according to the method supplied by the manufacturer to a final concentration of 10 mg protein/mL Sepharose. Columns were prepared with 17 mL gel, neutralized in 0.1 mol/L NaHCO₃-0.5 mol/L NaCl buffer (pH 8.3), and equilibrated in 0.15 mol/L NaCl (pH 7.4). Human serum (40 mL) (a gift from the blood bank, Karolinska Hospital) was applied to the column and the flow-through fractionated. Fractions were assayed for AT III activity as described below and pooled.

**Results**

Characterization of Human SMC Cultures

The cultured cells were characterized as SMCs by a typical "hill-and-valley" growth pattern at confluency, and in addition, positive immunostaining with anti-SMC α-actin (Fig 1, a and b). These observations were consistent throughout the numbers of passages used in the experiments. No staining was observed with antibodies against a human fibroblast cell surface antigen or von Willebrand factor, demonstrating the absence of contaminating fibroblasts or endothelial cells in the cultures (not shown).

Mitogenic Activity of Thrombin in Human SMCs

Human thrombin was analyzed for its ability to induce DNA synthesis and cell proliferation in subconfluent human SMC cultures. The lag phase required for thrombin to initiate DNA synthesis as evaluated by [³H]thymidine incorporation was found to be delayed compared with 5% NCS and 10 ng/mL of PDGF B. The peak values of [³H]thymidine incorporation for 5% NCS and PDGF B were detected at 36 hours, but a progressive increase in DNA synthesis in cultures stimulated with 10 nmol/L thrombin was observed throughout an incubation period of 50 hours (Fig 2A). A concentration-dependent induction of DNA synthesis by thrombin was demonstrated, and the half-maximal effect was obtained at 0.1 to 1 nmol/L (Fig 2B). The ability of thrombin to mediate cell proliferation was demonstrated by incubating serum-starved SMC cultures with various concentrations (1 to 100 nmol/L) of thrombin and subsequent cell counting (Fig 2C). Thrombin 100 nmol/L resulted in the highest increase in cell number.
during the first 4 days of culture. However, a significant decrease in cell number was observed after 6 days of incubation, indicating a toxic effect of this concentration. In comparison with 10% NCS, the ability of 10 nmol/L thrombin to promote cell proliferation was limited (1.5 versus 4.8 cell doublings in 6 days; not shown). On the basis of these results, further experiments were performed at a thrombin concentration of 10 nmol/L, and DNA synthesis was analyzed after an incubation period with $[^3H]$thymidine for 48 hours.

**Inhibition of the Mitogenic Activity of Thrombin in Human SMCs**

To evaluate the effect of the main physiological inhibitor of thrombin coagulant activity, AT III, thrombin was allowed to form complexes with AT III (TAT) at equimolar concentrations and with a 10-fold excess of AT III. In the absence of heparin, the formation of TAT complexes is progressive; therefore, incubation of AT III with thrombin was done for 5 hours at 37°C. Thrombin was incubated under similar conditions in the absence of AT III without detectable loss of enzymatic or mitogenic activity. After complex formation, measurement of residual thrombin enzymatic activity with the aid of the synthetic chromogenic substrate showed that in equimolar dilution with AT III, approximately 0.3 nmol/L free thrombin remained, whereas no thrombin enzymatic activity was detected in a 10-fold molar excess of AT III. The effect of TAT complexes on the initiation of DNA synthesis and cell proliferation in...
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Human SMC cultures was analyzed and compared with the effect of r-hirudin. Complex formation with AT III resulted in a marked decrease in the mitogenic activity of thrombin, comparable to the inhibition by r-hirudin (Fig 3A). If thrombin and AT III were added simultaneously to the cell culture medium, the inhibition of thrombin-induced mitogenic activity was less pronounced (Fig 3A).

**Effect of Heparin on Thrombin-Induced DNA Synthesis**

Since formation of TAT complexes is greatly enhanced in the presence of heparin, we studied the effect of heparin on the inhibitory action of AT III on thrombin-induced DNA synthesis in SMCs. The inhibition of thrombin mitogenic activity by AT III was greatly increased in the presence of heparin, whereas heparin alone failed to inhibit thrombin (Fig 4).

Measurements of thrombin enzymatic activity in the same test samples showed that the enzymatic activity of thrombin was unaffected by heparin alone, whereas an almost complete inhibition was seen in the presence of AT III and heparin (not shown).

**Inhibition of Thrombin-Induced DNA Synthesis in the Presence of Serum**

Induction of DNA synthesis in human SMCs by thrombin and the effects of heparin were also investigated in experiments performed in the presence of normal human serum (HuS), AT III-depleted HuS, or NCS. The concentration of AT III in HuS was determined before and after depletion of AT III by immunoaffinity chromatography (Table). HuS 0.5% and AT III-depleted HuS 0.5% caused an increased DNA synthesis in SMCs and potentiated the effect of thrombin to a similar extent (Fig 5A). Heparin inhibited the induction of DNA synthesis by 0.5% and 5% (not shown) HuS, AT III-depleted HuS, and NCS to a similar extent in concentrations ranging from 1 to 100 µg/mL (Fig 5B). DNA synthesis after thrombin stimulation in the presence of either 0.5% HuS or NCS was reduced by heparin, but the ability of thrombin to induce DNA synthesis in AT III-depleted HuS was unaffected by heparin. At high (50 to 100 µg/mL) heparin concentrations, a significantly more potent effect in 0.5% NCS than in 0.5% HuS was observed, whereas no significant differences were obtained in low (1 to 10 µg/mL) heparin concentrations. The more potent effect of heparin at high concentrations was also related to a higher AT III concentration in NCS (7 versus 2.8 nmol/L; Table and Fig 5C).

**Concentrations of AT III in Human Serum, Newborn Calf Serum, and Human Serum Depleted of AT III by Immunoaffinity Chromatography**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>AT III Concentration (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum</td>
<td>560</td>
</tr>
<tr>
<td>Newborn calf serum</td>
<td>1400</td>
</tr>
<tr>
<td>AT III-depleted human serum</td>
<td>≤10</td>
</tr>
</tbody>
</table>

AT III indicates antithrombin III.
The enzymatic activity of thrombin in the presence of the different sera used after addition of heparin was correlated with the inhibitory effects on mitogenic activity and the concentration of AT III. The enzymatic activity of thrombin measured with a chromogenic substrate was only slightly reduced by heparin in AT III–depleted serum, whereas 0.5% HuS had an intermediate effect, and 0.5% NCS efficiently inhibited thrombin (Fig 5D).

Discussion

The diverse functional properties of thrombin have recently led to an increased interest in a possible role of this enzyme in postclotting events occurring after vascular injury, particularly the growth of vascular SMCs. The enzymatically active form of thrombin, α-thrombin, has been demonstrated to act as a mitogen for SMCs by a number of investigators. Recently, this was also demonstrated for cultured human SMCs, a finding confirmed by our investigation. Attempts to inhibit the mitogenic activity of thrombin have previously been focused on nonphysiological thrombin inhibitors, whereas endogenous thrombin inhibitors have attracted little attention. AT III is the most important physiological inhibitor of thrombin coagulant activity. TAT complex formation is catalyzed by glycosaminoglycans in the vessel wall extracellular matrix and at the surface of cells or by exogenously administered heparin, which induces conformational changes in the AT III molecule. AT III was also found to abolish the mitogenic activity of thrombin when complex formation between the two was allowed. Addition of heparin strongly increased the inhibitory activity of AT III on thrombin-induced DNA synthesis in SMCs. The potentiating effect of exogenous heparin on AT III–mediated thrombin inhibition indicates that cell- or matrix-bound glycosaminoglycans were insufficient in catalyzing this interaction, since addition of AT III and thrombin simultaneously resulted in only a 25% to 30% inhibition of thrombin activity.

Even though AT III is consumed during clotting, sufficient amounts remain in serum to support thrombin inhibition. In 0.5% serum, thrombin was inhibited by increasing concentrations of heparin. At high heparin concentrations, this inhibition was related to the AT III concentrations in the different preparations of sera used. NCS 0.5%, which was estimated to contain 7 nmol/L AT III, inhibited thrombin more efficiently than 0.5% human serum, containing only 2.8 nmol/L AT III. This biological variation may explain the different re-
results of Herbert et al, who were able to demonstrate only limited inhibition of thrombin-induced DNA synthesis by increasing heparin concentrations diluted in 0.5% FCS. In support of our findings, McNamara et al recently reported a reduction in thrombin-induced DNA synthesis in rat aortic SMCs when experiments were performed in the presence of 0.2% FCS. Our observations demonstrate the necessity of controlling AT III concentrations in the medium when evaluating thrombin activity in cell culture models. The importance of AT III in regulating the mitogenic activity of thrombin was further demonstrated by depletion of AT III from serum, which resulted in a complete loss of inhibitory activity by heparin on thrombin-induced DNA synthesis.

Interestingly, heparin did not inhibit thrombin-induced DNA synthesis in the absence of serum. This is in contrast to the well-established inhibitory effect of heparin on serum- or growth factor–induced DNA synthesis. These properties of heparin have been suggested to be a result of extracellular growth factor clearance, such as in the case of basic fibroblast growth factor. Alternative explanations include potentiation of transforming growth factor–β activity or a direct effect on intracellular growth-regulatory responses.

Thrombin-induced cell proliferation may be partially due to induction of endogenous PDGF A expression in SMCs. Since PDGF and other growth factors interact with heparin and heparan sulfate proteoglycans, heparin may interfere with autocrine responses induced by thrombin. However, our results outline a neutralizing effect of heparin on thrombin activity and endogenous growth factors, since heparin in the absence of AT III was unable to inhibit either DNA synthesis or clot formation induced by thrombin. Rather, the results indicate that the growth-stimulatory effects of thrombin on SMCs are mediated by heparin-resistant mechanisms in a different manner than serum or growth factors. This is in contrast to previous findings in rat SMCs, in which heparin was found to inhibit cell proliferation and neointima formation in an AT III–independent manner. However, AT III levels and thrombin concentrations were not controlled in these experiments, and the results may have been due to interactions between heparin and serum factors other than AT III and thrombin. This was also supported by our findings that heparin did inhibit DNA synthesis induced by serum even after AT III depletion.

The inhibition of thrombin-induced DNA synthesis after the catalytic site of thrombin was blocked with AT III or t–hirudin also demonstrates that the enzymatic activity of thrombin is related to its potency as a mitogen for SMCs, which has been demonstrated previously in rabbit SMCs. This relation has been questioned previously on the basis of results from experiments with enzymatically inactivated thrombin on bovine SMCs. Conversely, others have not been able to demonstrate either mitogenic activity or induction of PDGF A mRNA by nonenzymatic thrombin. Moreover, the recent demonstration of a proteolytic cleavage mechanism involved in thrombin receptor activation indicates that the catalytic site of thrombin is associated with receptor stimulatory function. Recently, McNamara et al also reported that thrombin-induced DNA synthesis in rat aortic SMCs was dependent on the enzymatic activity of thrombin. In addition, they demonstrated the expression of mRNA in these cells, which in Northern blot analysis hybridized with the human thrombin receptor cDNA identified by Vu et al.

Binding of AT III to the vessel surface is facilitated by endogenous sulfated glycosaminoglycans present both in the intact endothelium and in subendothelial layers. Addition of AT III to the injured vessel wall results in an enhanced inhibition of thrombin. After balloon injury of the rabbit aorta, enzymatically active thrombin is localized to the vessel wall, but treatment of the animals with AT III reduces the amount of enzymatically active thrombin on the vessel wall. At the same time, AT III is shown to accumulate on the exposed subendothelium. These results indicate that AT III is localized at the site of vascular injury, where it may take part in the inhibition of thrombin. Conversely, AT III has been reported to lack an inhibitory effect on thrombin when bound to the extracellular matrix formed by bovine corneal endothelial cells, and its growth-stimulatory effect on SMCs is reported to be retained. Thus, the AT III/heparin–mediated inhibition of thrombin-induced SMC proliferation may be restricted to fluid-phase thrombin. However, the possibility that other thrombin inhibitors, such as heparin cofactor II, regulate thrombin activity in the pericellular matrix of SMCs must also be remembered.

Thrombosis and SMC proliferation in neointima formation are two critical consequences of reconstructive vascular procedures. Elucidating the properties of factors with regulatory functions in both of these processes may lead to new prospects in therapeutic intervention of restenotic lesions. A role for thrombin in both of these complications has already been suggested, and the present report indicates that AT III may have dual functions, counteracting both the coagulant and the mitogenic effects of thrombin. Further investigations are required in animal models and in patients with subnormal serum levels of AT III to verify a regulatory function of AT III in lesion formation after vascular injury.

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References

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