Antiangiogenic Antithrombin Blocks the Heparan Sulfate-dependent Binding of Proangiogenic Growth Factors to Their Endothelial Cell Receptors

EVIDENCE FOR DIFFERENTIAL BINDING OF ANTIANGIOGENIC AND ANTICOAGULANT FORMS OF ANTIITHROMBIN TO PROANGIOGENIC HEPARAN SULFATE DOMAINS

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The anticoagulant serpin antithrombin acquires a potent antiantiangiogenic activity upon undergoing conformational alterations to cleaved or latent forms. Here we show that antithrombin antiantiangiogenic activity is mediated at least in part through the ability of the conformationally altered serpin to block the proangiogenic growth factors fibroblast growth factor (FGF)-2 and vascular endothelial growth factor (VEGF) from forming signaling competent ternary complexes with their protein receptors and heparan sulfate co-receptors on endothelial cells. Cleaved and latent but not native forms of antithrombin blocked the formation of FGF-2-FGF receptor-1 ectodomain-heparan sulfate ternary complexes, and the dimerization of these complexes in solution and similarly inhibited the formation of FGF-2-heparin binary complexes and their dimerization. Only antiantiangiogenic forms of antithrombin likewise inhibited 125I-FGF-2 binding to its low affinity heparan sulfate co-receptor and blocked FGF receptor-1 autophosphorylation and p42/44 MAP kinase phosphorylation in cultured human umbilical vein endothelial cells (HUVECs). Moreover, treatment of HUVECs with heparinase III to specifically eliminate the FGF-2 heparan sulfate co-receptor suppressed the ability of antiantiangiogenic antithrombin to inhibit growth factor-stimulated proliferation. Antiantiangiogenic antithrombin inhibited full-length VEGF165 stimulation of HUVEC proliferation but did not affect the stimulation of cells by the heparin-binding domain-deleted VEGF121. Taken together, these results demonstrate that antiantiangiogenic forms of antithrombin block the proangiogenic effects of FGF-2 and VEGF on endothelial cells by competing with the growth factors for binding the heparan sulfate co-receptor, which mediates growth factor-receptor interactions. Moreover, the inability of native antithrombin to bind this co-receptor implies that native and conformationally altered forms of antithrombin differentially bind proangiogenic heparan sulfate domains.

Angiogenesis, the growth of new capillaries from pre-existing vessels, is a key physiologic process whose dysregulation underlies many diseases (1). It is particularly important for the transition of tumors from a dormant state to a malignant state, because new vessels supply oxygen, essential nutrients, and growth factors that allow the tumor mass to expand. This expectation has led to the idea that angiogenesis inhibitors may be useful antitumour drugs (2). A number of naturally occurring angiogenesis inhibitors have been identified as potential antitumor therapeutic agents that are derived by modification of endogenous proteins, e.g. angiotatin is a fragment of plasminogen (3), endostatin is a fragment produced by proteolytic cleavage of collagen XVIII (4), and the serpin antithrombin acquires antiantiangiogenic activity upon undergoing conformational alterations induced by mild heating or protease cleavage (5).

Interestingly, whereas a number of serpins, such as maspin, pigment epithelium-derived factor, and kallistatin, have been shown to possess antiantiangiogenic activity (6–8), only in the case of antithrombin does this activity require conformational changes in the protein (5, 9). Crystal structures of cleaved and latent antiantiangiogenic forms of antithrombin have revealed that the conformational alterations involve the insertion of the N-terminal end of an exposed reactive loop into the center of the major β-sheet of the protein (10, 11). Both conformationally altered antithrombin forms are produced under physiologic conditions, and therefore their antiantiangiogenic activity could have physiologic relevance (12, 13).

It has been well documented that conformationally altered forms of antithrombin regulate angiogenesis on cultured endothelial cells stimulated by heparin-binding growth factors, e.g. FGF-2 and VEGF (5, 9, 14). Previous results including those from our group have demonstrated that cleaved and latent forms of antithrombin exert their antiantiangiogenic effects by inducing cell apoptosis (9, 15, 16), inhibiting endothelial cell cycle progression (17), and suppressing the expression of the proangiogenic heparan sulfate proteoglycan (HSPG) perlecan, as well as other proangiogenic genes in cultured HUVECs (16, 17). However, the molecular mechanisms of antithrombin antiantiangiogenic action still remain to be defined.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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We recently showed that the heparin-binding site of antithrombin, which mediates heparin binding and activation of the anticoagulant function of the serpin, is also a critical mediator of the anticoagulant activity of cleaved and latent forms of the protein (18). This finding in conjunction with reports that other endogenous angiogenesis inhibitors such as endostatin and kallistatin bind heparin or heparan sulfate to produce their anticoagulant effects (19, 20) suggested that anticoagulant forms of antithrombin similarly might act by binding endothelial cell-associated heparan sulfate molecules. Such binding could produce anticoagulant effects if it blocked the binding of the proangiogenic growth factors, FGF-2 and VEGF, to their HSPG co-receptors (21, 22).

In the present study we provide data to support such a mechanism of antithrombin anticoagulant action. We thus show that anticoagulant but not native forms of antithrombin inhibit FGF-2 binding to immobilized heparin, block FGF-2 self-dimerization mediated by heparin, and inhibit heparin-dependent binding of FGF-2 to FGF receptor-1 ectodomains as well as the dimerization of the FGF-2–FGF receptor-1 complex in solution (23, 24). Heparin molecules lacking the anticoagulant binding sequence for native antithrombin are as effective as unfractionated heparin in mediating these effects. Anticoagulant antithrombin is further shown to inhibit 1125-FGF-2 binding to its HSPG co-receptor and suppress HSPG-dependent signaling and growth by FGF-2 or VEGF receptor complexes in cultured HUVECs (21, 22, 25). Treatment of HUVECs with heparinase III to eliminate the HSPG co-receptor for FGF-2 is shown to abolish the inhibitory effect of latent antithrombin on growth factor-stimulated cell proliferation or capillary tube formation (26). Taken together, these findings demonstrate that the anticoagulant function of antithrombin is mediated at least in part by blocking FGF-2 or VEGF binding to their HSPG co-receptors. This blockade results from the preferential binding of anticoagulant forms of antithrombin to proangiogenic sequences or domains in heparin or heparan sulfate that are distinct from the anticoagulant sequence recognized by native antithrombin (27).

**EXPERIMENTAL PROCEDURES**

**Antithrombin**—Native, latent, and neutrophil elastase-cleaved forms of human plasma-derived antithrombin were prepared as described previously (17).

**Heparin**—Unfractionated heparin from porcine intestinal mucosa and with an average molecular weight of ∼15,000 was obtained from Sigma. A fractionated heparin with low affinity for native antithrombin because of the absence of the antithrombin-binding pentasaccharide sequence and containing ∼26 saccharides (Mₙ = ∼7900) was isolated from unfractionated heparin by size exclusion and repeated antithrombin–agarose affinity chromatography steps as previously described (28, 29).

**Cell Culture**—HUVECs were purchased from Cascade Biologics (Portland, OR) and cultured in Medium-200 containing 10% fetal calf serum plus growth supplements and 1% antibiotics (penicillin and streptomycin) (Cascade Biologics) at 37 °C, 5% CO₂ in air. HUVECs were used within 10 passages.

**FGF-2-Heparin Binding Assay**—Heparin immobilized on acrylic beads (Sigma) was washed twice with Hanks’ balanced saline solution (HBSS; Invitrogen), suspended in an equal volume of HBSS, and then 20 μl of the bead slurry was incubated with 10 ng/ml of FGF-2 (recombinant human form; Invitrogen) and varying amounts of native or cleaved antithrombin in a 50-μl total reaction volume for 30 min at room temperature. The heparin beads were washed with 0.5 ml of HBSS buffer three times to remove free FGF-2, and FGF-2 bound to heparin beads was released by suspending the beads in SDS electrophoresis buffer and boiling for 5 min. Bound FGF-2 samples were then subjected to SDS-PAGE (12% gel) and analyzed by immunoblotting. Protein on the polyacrylamide gel was first transferred onto Immobilon TM-P membranes (Millipore Corp., Bedford, MA). The membranes were blocked by incubation at 25 °C for 60 min in 5% milk in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20), incubated with mouse anti-FGF-2 antibodies (Oncogene, Cambridge, MA) for 16 h at 4 °C, washed in TBST, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) for 60 min at 25 °C. The immunoreactive proteins were detected by the enhanced chemiluminescence detection system according to the manufacturer’s instructions (Amersham Biosciences). Protein bands on immunoblots were quantitated with a Kodak Image Station 440-CF (Kodak, Rochester, NY).

**FGF-2 Binding to FGF Receptor-1 Ectodomain**—The recombinant ectodomain of FGF receptor-1 isoform IIIc conjugated to alkaline phosphatase (FGFR1c-AP) was a generous gift from Dr. Alan Rapraeger (University of Wisconsin-Madison). The binding assay contained 10 ng/ml of FGF-2, 10 ng/ml FGFR1c-AP, 0.1 μg/ml unfractionated heparin, and varying amounts of native or cleaved antithrombin in HBSS in 200 μl of total volume (23). One set of samples was incubated for 30 min at room temperature followed by adding 10 μl of anti-alkaline phosphatase antibody coupled to agarose beads (sigma) and incubating further 2 h to immunoprecipitate FGFR1c-AP complexes with FGF-2. A second set of samples was treated with chemical cross-linker (see below) after the initial incubation and incubated an additional 30 min prior to immunoprecipitating the receptor in order to detect complex formation with antithrombin. Free FGF-2 and antithrombin were removed by washing the immobilized antibody beads twice with 1 ml of HBSS buffer. FGF-2 noncovalently bound to the receptor, and cross-linked antithrombin–receptor complexes were released from the beads and resolved by SDS-PAGE (12%) as in the FGF-2-heparin bead binding experiments. Resolved proteins were then detected by immunoblotting with mouse anti-FGF-2 or sheep anti-antithrombin (The Binding Site, Birmingham, UK) primary antibodies together with appropriate species-specific horseradish peroxidase-conjugated secondary anti-IgG antibodies as described above.

**Chemical Cross-linking of FGF-2 and FGF-2-FGFR1c-AP Complexes**—To measure FGF-2 multimer formation in the presence of heparin, FGF-2 (10 ng/ml) was incubated in HBSS in the absence or presence of 0.5 μg/ml unfractionated heparin or 0.1 μg/ml ∼26-saccharide heparin with low antithrombin affinity and with varying levels of native or cleaved antithrombin as in the heparin bead binding assay. FGF-2-FGFR1c-AP complex formation was measured by incubating FGF-2, FGFR1c-AP, and heparin in the presence or absence of anti-
thrombin in HBSS for 30 min as described above for the binding assay. Cross-linking of both types of complexes was initiated by adding bis-sulfosuccinimidyl-suberate (Pierce) to a final concentration of 0.01 mg/ml and incubating the mixture for a further 10 min. The reaction was quenched by adding ethanolamine-HCl, pH 8.0 (final concentration, 10 mM), and incubating for 15 min. Cross-linked protein complexes were then resolved by SDS-PAGE (15% gel for FGF-2 multimers and 8% gel for FGF-2-FGFR1c-AP complexes). Cross-linked FGF-2 multimers and FGF-2-FGFR1c-AP complexes were detected by immunoblot analysis using anti-FGF-2 antibody as above.

Effect of Heparinases on Antithrombin Inhibition of HUVEC Proliferation or Capillary Tube Formation—For cell proliferation assays, HUVECs were cultured in starving medium (Medium-200 containing 0.5% fetal bovine serum) for 24 h in a 96-well plate. The cells were incubated with 1 unit/ml of either heparinase I, heparinase II, or heparinase III (Sigma) for 4 h at 37 °C (30). The cells were washed with starving medium twice before they were treated with antithrombin and FGF-2. Cell proliferation rates were quantified after another 48-h incubation by adding 20 µl of Cell-Title solution (Promega, Madison, WI), incubating for 1–4 h and measuring the A490 as described previously (18). For capillary tube formation assays, the cells were treated with heparinases as above, then the cells were suspended in medium plus FGF-2 and seeded onto Matrigel-coated wells, and after allowing attachment cells were incubated in the absence or presence of different forms of antithrombin as described (18).

Antithrombin Effects on VEGF-stimulated HUVEC Proliferation—HUVECs were starved in Medium-200 supplemented with 0.5% fetal bovine serum for 12 h. They were then treated with either VEGF165 or VEGF121 (Invitrogen) together with native or cleaved forms of antithrombin and incubated for 48 h. Cell proliferation rates were quantified as above with Cell-Title solution.

RESULTS

Effect of Antithrombin on FGF-2 Binding to Heparin—The proangiogenic effects of FGF-2 are mediated by binding to an endothelial cell protein receptor and heparan sulfate proteoglycan co-receptor (26, 31). To test whether antiangiogenic antithrombin might block the proangiogenic effects of FGF-2 by competitively binding to its heparan sulfate co-receptor, we determined whether the antiangiogenic cleaved form of antithrombin could inhibit the binding of FGF-2 to heparin, an established model for the heparan sulfate co-receptor (22). FGF-2 binding to heparin immobilized on acrylic beads was assayed in the absence and presence of antiangiogenically active (cleaved) and inactive (native) forms of antithrombin. FGF-2-heparin complexes were collected by centrifugation and subjected to immunoblot analysis with anti-FGF-2 antibody. FGF-2 bound with high affinity to the immobilized heparin (Fig. 1, lane 2), and this binding could be almost completely inhibited by cleaved antithrombin in a dose-dependent manner (lanes 6–8). By contrast, native antithrombin failed to inhibit FGF-2 binding to heparin but rather modestly stimulated the binding at equivalent doses (lanes 3–5).

Effect of Antithrombin on Heparin-mediated FGF-2 Dimerization—Heparin binds to FGF growth factors in a multivalent manner, resulting in their oligomerization (23, 32). To assess whether cleaved antithrombin could block the heparin-
dependent oligomerization of FGF-2, FGF-2 was incubated with heparin in the absence or presence of native and cleaved forms of antithrombin, and then oligomerization of FGF-2 was analyzed by chemical cross-linking followed by SDS-PAGE and immunoblotting with anti-FGF-2. Heparin was observed to promote the formation of an FGF-2 dimer (Fig. 2, lanes 1 and 5), and this dimerization was abrogated by cleaved antithrombin in a dose dependent fashion (lanes 6–8). Native antithrombin did not inhibit the dimerization and appeared to slightly enhance the formation of FGF-2 dimers (lanes 2–4), consistent with the ability of native antithrombin to promote FGF-2 binding to heparin (Fig. 1). Heparin lacking the anticoagulant binding sequence for native antithrombin (0.1 μg/ml), Molecular mass markers are in kilodaltons.

To determine whether cleaved antithrombin could inhibit the heparin-dependent binding of FGF-2 to the FGF receptor, FGF-2 was incubated with a recombinant FGFR1 ectodomain-alkaline phosphatase fusion protein (FGFR1c-AP) in the presence of heparin and different forms of antithrombin. The FGFR1c-AP receptor was then immunoprecipitated with anti-alkaline phosphatase (anti-AP) antibody and then detected by immunoblot (IB) analysis using anti-FGF-2 antibody (upper panel) or anti-antithrombin (anti-AT) antibody (lower panel) after 12% SDS-PAGE as described under “Experimental Procedures.”

FIGURE 1. Cleaved antithrombin inhibits FGF-2 binding to immobilized heparin. FGF-2 was incubated with immobilized heparin beads in the absence and presence of different forms of antithrombin, and FGF-2 bound to the beads was detected by immunoblotting as described under “Experimental Procedures.” FGF-2 bound to heparin beads is shown for incubations of beads in buffer (lane 1), with FGF-2 alone (lane 2), with FGF-2 and native antithrombin (AT; lanes 3–5), and with FGF-2 and cleaved antithrombin (lanes 6–8).

FIGURE 2. Cleaved antithrombin blocks the heparin-mediated dimerization of FGF-2. FGF-2 (10 ng/ml) was incubated alone (lane 5) or with heparin (lane 1) and increasing concentrations of native antithrombin (AT, lanes 2–4) or cleaved antithrombin (lanes 6–8) for 1 h at 24 °C. Bis-sulfosuccinimidylsuberate was then added to initiate cross-linking, the reaction was quenched with ethanolamine, and cross-linked FGF-2 was analyzed by 15% SDS-PAGE and immunoblotting with anti-FGF-2 antibodies as described under “Experimental Procedures.” The upper panel shows experiments conducted with unfraccionated heparin (0.5 μg/ml), and the lower panel shows experiments conducted with a fractionated ~7900 M₆ heparin lacking the anticoagulant binding sequence for native antithrombin (0.1 μg/ml). Molecular mass markers are in kilodaltons.

FIGURE 3. Cleaved antithrombin inhibits the formation of FGF-2-FGF receptor-heparin ternary complexes. Recombinant FGFR1c-AP (10 ng/ml) was incubated with 10 ng/ml FGF-2 in the absence (lane 1) or presence (lanes 2–8) of 0.1 μg/ml heparin and increasing concentrations of native antithrombin (lanes 3–5) or cleaved antithrombin (lanes 6–8) and either in the absence or presence of chemical cross-linker. Noncovalent complexes of FGF-2 or covalent complexes of antithrombin with FGFR1c-AP were immunoprecipitated (IP) with anti-alkaline phosphatase (anti-AP) antibody and then detected by immunoblot (IB) analysis using anti-FGF-2 antibody (upper panel) or anti-antithrombin (anti-AT) antibody (lower panel) after 12% SDS-PAGE as described under “Experimental Procedures.”

FIGURE 4. Cleaved and latent antithrombins inhibit the dimerization of FGF-2-FGF receptor-heparin ternary complexes. FGF-2 was incubated with FGFR1c-AP in the presence of heparin and different forms of antithrombin, and the FGF-2-FGFR1c-AP complexes formed were cross-linked with bis-sulfosuccinimidyl suberate, resolved by 8% SDS-PAGE and detected by immunoblot analysis with anti-FGF-2 antibody as described under “Experimental Procedures.” Incubations of FGF-2 with heparin were done in the absence (lanes 1 and 7) or presence of FGFR1c-AP (lanes 2–6 and 8–12) and with 25 μg/ml of either native antithrombin (N, lanes 3 and 9), cleaved antithrombin (C, lanes 4 and 10), latent antithrombin (L, lanes 5 and 11), or a mixture of cleaved and native antithrombins (lanes 6 and 12). The experiment in the left panel was done with unfraccionated heparin, and that in the right panel was done with fractionated heparin with low antithrombin affinity. Molecular mass markers are in kilodaltons.
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To determine whether cleaved antithrombin inhibits the heparin-dependent binding of FGF-2 to FGFR1c-AP complex bands (lanes 3 and 9). However, the presence of cleaved or latent forms of antithrombin blocked the appearance of the complex bands (lanes 4, 5, 10, and 11). The addition of native antithrombin did not affect the ability of cleaved antithrombin to block complex formation (lanes 6 and 12).

To determine whether cleaved antithrombin inhibits the heparin-dependent binding of FGF-2 to FGFR1c-AP by competitively binding to FGFR1c-AP, we added cross-linker to the FGFR1c-AP receptor mixtures in the experiment of Fig. 3 and analyzed the immunoprecipitated receptor for cross-linked antithrombin by immunoblotting with an anti-antithrombin antibody. Antithrombin was detected in the immunoprecipitated FGFR1c-AP when cleaved antithrombin was added to block the heparin-dependent binding of FGF-2 to FGFR1c-AP (Fig. 3, lower panel, lanes 6–8). However, no antithrombin was detected in the immunoprecipitate when native antithrombin was present, conditions that did not affect FGF-2 binding to the immunoprecipitated receptor (lanes 3–5). Control experiments showed that cleaved antithrombin was not immunoprecipitated with the receptor in the presence of FGF-2 when heparin was absent (not shown). These findings support the idea that cleaved antithrombin specifically blocks the formation of FGF-2-FGFR receptor-heparin ternary complexes by directly competing with FGF-2 for the heparin moiety of the complexes.

Effect of Antithrombin on FGF-2 Binding to HUVECs—To determine whether the inhibitory effect of antiangiogenic antithrombin on the heparin-mediated binding of FGF-2 to the FGFR1c-AP receptor in solution was also observable in cultured endothelial cells, we tested the effects of native and antiangiogenic forms of antithrombin on 125I-FGF-2 binding to both the low affinity heparin sulfate co-receptor and to high affinity FGF receptors on cultured HUVECs (22). Because cleaved and latent forms of antithrombin behave indistinguishably with respect to their heparin affinity and antangiogenic activities in several types of assays (9, 14, 18), these experiments were performed with latent antithrombin. Binding of 125I-FGF-2 to the low affinity HSPG co-receptor, detected by a high salt elution of the glycosaminoglycan-bound growth factor, was slightly enhanced by native antithrombin but was significantly inhibited by latent antithrombin in a dose-dependent manner, i.e. up to 60% inhibition of binding was observed at the highest level tested (50 μg/ml) (Fig. 5, upper panel). The inhibitory effect of the latent serpin was not significantly affected by including native antithrombin at levels as high as the latent serpin. 125I-FGF-2 bound to high affinity FGF receptors on the cells, detected by elution of residual cell-bound FGF-2 with a low pH and high salt buffer wash, was insignificantly affected by the addition of native antithrombin and only marginally decreased in the presence of latent antithrombin in a manner minimally dependent on the dose or the addition of native antithrombin (Fig. 5, lower panel).

Effect of Antithrombin on FGF-2 Signaling through FGF Receptor-1 on HUVECs—Cell surface heparan sulfate promotes fibroblast growth factor binding to specific FGF receptors which induce receptor dimerization, autophosphorylation, and signaling (21, 22, 32). We previously showed that receptor autophosphorylation and phosphorylation of the downstream target of the mitogenic signaling cascade p42/44 MAP kinase induced by FGF-2 binding to the FGF-2 receptor and heparan sulfate co-receptor was inhibitable by antiangiogenic antithrombin at a single 20 μg/ml dose (18). To confirm this observation and establish its dependence on antithrombin dosage, we incubated quiescent HUVECs with varying amounts of cleaved and native forms of antithrombin prior to stimulating the cells with FGF-2. The cells were then lysed and subjected to SDS-PAGE and immunoblot analysis for FGF receptor-1 autophosphorylation (34, 35) (Fig. 6). Phosphorylated FGFR1 was enhanced by stimulation of HUVECs with FGF-2, and native antithrombin had minimal effects on the receptor autophos-
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Effects of Antiangiogenic Antithrombin on VEGF-stimulated HUVEC Growth—Interactions of HSPG with full-length VEGF$_{165}$ enhance VEGF binding to its receptor on the endothelial cell surface, whereas VEGF$_{121}$, an alternatively spliced variant of VEGF that lacks the heparin-binding domain, does not require cell surface HSPG for its mitogenic action (37). We therefore compared the inhibitory effects of cleaved antithrombin on full-length VEGF$_{165}$ and truncated VEGF$_{121}$ stimulation of HUVEC proliferation (Fig. 8). Cleaved antithrombin only inhibited HUVEC proliferation stimulated by VEGF$_{165}$ (lanes 4–6) and not that stimulated by VEGF$_{121}$ (lanes 7–9), indicating that only the heparan sulfate-dependent stimulation of cells by VEGF was inhibitable by antiangiogenic antithrombin.

DISCUSSION

We recently showed that the heparin-binding site of antithrombin is essential for mediating the antiangiogenic activity of cleaved and latent forms of the serpin (18), strongly implicating an endothelial cell heparan sulfate receptor or co-receptor in this activity. In the present study we have provided evidence to support the idea that cleaved and latent antithrombins produce their antiangiogenic effects by competing with the proan-

Effects of Heparinase on Antithrombin Inhibition of Proliferation and Capillary Tube Formation in FGF-2-stimulated HUVECs—The involvement of heparan sulfate on cell surfaces or the extracellular matrix in FGF-2 and VEGF action has been best demonstrated by the effects of removal or desulfation of heparan sulfate by heparinasins (21, 22, 26, 35). We therefore treated HUVECs with bacterial heparinasins I, II, and III before exposing them to FGF-2 and latent antithrombin. Pretreatment with heparinasins I and II did not significantly affect the anti-proliferative effects of latent antithrombin on HUVECs stimulated with FGF-2, but predigestion with heparinase III abolished the inhibitory effect of latent antithrombin on FGF-2 stimulated HUVEC proliferation (Fig. 7). It should be noted that the ability of FGF-2 to stimulate HUVEC proliferation was only modestly affected by treatment with any of the heparinasins, an observation previously made and attributed to the heparan sulfate-independent binding of FGF-2 to FGF receptors on the cells (36).

Similar findings were made when the effects of native and latent antithrombins on HUVECs treated with heparinasins I, II, and III were examined in a capillary tube formation assay. Heparinasins I and II had no effect on the FGF-induced differentiation of HUVECs into capillary tubes on Matrigel or on the inhibitory effect produced by latent antithrombin but not native antithrombin on the formation of capillary tubes (supplemental Fig. S1). Although heparinase III treatment was observed to reduce capillary tube formation induced by growth factor, both native and latent antithrombins produced no discernable effects on the extent of tube formation observed. Heparinase III treatment of HUVECs thus causes a loss of the inhibitory effect of latent antithrombin in this angiogenesis assay.

Effects of Heparinase on Antithrombin on FGF-2-dependent FGFR-1 autophosphorylation. HUVECs were starved in 0.5% FBS medium for 12 h, preincubated with increasing amounts of native antithrombin (AT, lanes 3–5) or cleaved antithrombin (lanes 6–8) for 2 h, and then stimulated with FGF-2 (15 ng/ml) for 15 min. After washing, the cells were lysed and immunoprecipitated with anti-FGFR1 antibody, and immunoprecipitates were subjected to 10% SDS-PAGE and analyzed for FGFR-1 autophosphorylation by immunoblotting with pY-20 antibody. Total FGFR1 protein was determined by stripping the membrane and immunoblotting with anti-FGFR-1 antibodies. Further details are provided under "Experimental Procedures."

FIGURE 6. Effects of antithrombin on FGF-2-dependent FGFR-1 autophosphorylation. HUVECs were starved in 0.5% FBS medium for 12 h, preincubated with increasing amounts of native antithrombin (AT, lanes 3–5) or cleaved antithrombin (lanes 6–8) for 2 h, and then stimulated with FGF-2 (15 ng/ml) for 15 min. After washing, the cells were lysed and immunoprecipitated with anti-FGFR1 antibody, and immunoprecipitates were subjected to 10% SDS-PAGE and analyzed for FGFR-1 autophosphorylation by immunoblotting with pY-20 antibody. Total FGFR1 protein was determined by stripping the membrane and immunoblotting with anti-FGFR1 antibodies. Further details are provided under "Experimental Procedures."

FIGURE 7. Heparinase III abolishes the antiproliferative effect of antiangiogenic antithrombin on FGF-2-stimulated HUVECs. Starved HUVECs were pretreated with 1 unit/ml each of bacterial heparinase I (lanes 7–12), heparinase II (lanes 13–18), or heparinase III (lanes 19–24) for 4 h before stimulating with FGF-2 in the absence or presence of native antithrombin or latent antithrombin. After 48 h, the number of cells were determined colorimetrically as described under "Experimental Procedures." 100% control values correspond to ~10,000 cells/well. The data were derived from three independent experiments and are presented as the means with error bars reflecting the S.D. values.
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growth factors, FGF-2 and VEGF, for binding to low affinity heparan sulfate co-receptors and thereby attenuating the ability of the growth factors to bind their high affinity endothelial cell protein receptors and produce their mitogenic effects (21, 22).

Antiangiogenic forms of antithrombin were shown to inhibit FGF-2 binding to heparin and to suppress other FGF-2 interactions that depend on the FGF-2-heparin interaction in solution. The latter include the dimerization of FGF-2, the binding of FGF-2 to the FGF receptor-1 ectodomain, and the dimerization of the FGF-2-FGF receptor-1 ectodomain complex (23). Heparin was used as a surrogate for the endothelial cell heparan sulfate co-receptor for FGF-2 in these solution studies based on the established ability of heparin to substitute for the heparan sulfate co-receptor in cells expressing FGF receptors but lacking heparan sulfate proteoglycans (21–24, 31). The crystal structure of the FGF-2-heparin-FGF receptor-1 ectodomain complex has shown that heparin promotes the binding of FGF-2 to FGF receptors by bridging FGF-2 and its receptor in a 1:1:1 ternary complex and inducing the ternary complexes to dimerize to a 2:2:2 complex through additional heparin bridging interactions that enhance the receptor-receptor interaction (38). The inhibition of the heparin-dependent binding and dimerization of FGF-2-FGF receptor complexes by antiangiogenic antithrombin was associated with antithrombin binding to the receptor. Because antithrombin was not observed to bind the soluble receptor in the absence of heparin, and the receptor is known to interact with heparin in the absence of growth factor (31), our findings suggest that antiangiogenic antithrombin inhibits FGF-2 binding to the soluble FGF receptor by binding to heparin and thereby disrupting the formation of monomeric or dimeric ligand-receptor complexes that require heparin for stabilization.

The observations made in solution were shown to be relevant for the FGF-2-FGF receptor interaction on endothelial cells. Antiangiogenic antithrombin was thus found to dose-dependently inhibit FGF-2 binding to low affinity heparan sulfate receptors on endothelial cells as well as FGF receptor-1 auto-phosphorylation and p42/44 MAP kinase phosphorylation, which are dependent on FGF-2 binding to FGF receptor-1 on the cell surface (34, 35). Moreover, removal of the heparan sulfate co-receptor by treatment of HUVECs with heparinase III (21, 22, 26, 35) abrogated the inhibitory effect of antiangiogenic antithrombin on HUVEC proliferation or differentiation into capillary tubes. Removal of the heparan sulfate co-receptor for FGF-2 still allowed FGF-2 to stimulate cell proliferation through FGF receptors in agreement with previous findings (36, 39) but impaired the ability of FGF-2 to induce HUVECs to differentiate into capillary tubes. These observations suggest that antiangiogenic antithrombin binding to the heparan sulfate co-receptor blocks not only the heparan sulfate-dependent engagement of FGF-2 with its receptor but also any heparan sulfate-independent engagement of the FGF-2-receptor interaction. This could occur if antithrombin specifically bound heparan sulfate domains required for FGF-2-FGF receptor-heparan sulfate ternary complexes and thereby forced FGF-2 to bind heparan sulfate domains that do not support the formation of growth factor-receptor complexes (38, 40–44). Such nonproductive FGF-2-heparan sulfate binary complexes would not form in heparinase III-treated endothelial cells, allowing FGF-2 to engage the FGF receptor to an extent sufficient to stimulate cell proliferation. Together, our findings suggest that antiangiogenic antithrombin inhibits FGF-2 binding to its endothelial cell receptor by a mechanism similar to that found for the inhibition of its binding to the soluble receptor ectodomain, i.e. one that involves competition for an endothelial cell heparan sulfate co-receptor whose specificity is governed by the requirements for binding both FGF-2 and FGFR1 in a ternary complex.

Our findings additionally suggest that a similar mechanism can explain the ability of antiangiogenic antithrombin to inhibit VEGF angiogenic activity. This inhibition was thus found to depend on the presence of the heparin-binding domain of VEGF, implying that the effect of antithrombin similarly involved blocking the heparan sulfate co-receptor, which promotes VEGF binding to its high affinity receptor (37, 45). Again, the ability of antiangiogenic antithrombin to inhibit the heparan sulfate-dependent binding of full-length VEGF to its receptor must also antagonize any heparan sulfate-independent binding of the full-length growth factor to this receptor through the formation of nonproductive growth factor-heparan sulfate interactions as with FGF-2.

Importantly, native antithrombin was found to be unable to compete with the growth factors for binding the heparan sulfate co-receptor, implying that the heparin binding specificities of native and antiangiogenic forms of antithrombin are quite distinct. Although native antithrombin is known to bind to a specific pentasaccharide sequence in heparin and binds to immobilized heparin as well as to endothelial cell-associated heparan sulfates with high affinity through such sequences (27, 46, 47), this binding minimally affected FGF-2 binding to heparin or other FGF-2 interactions dependent on heparin binding, i.e. FGF-2 dimerization, FGF-2 binding to FGF receptor-1 ectodomain, and FGF-2-FGFR1 complex dimerization. Heparin molecules lacking the pentasaccharide sequence showed the same ability as unfractionated heparin to mediate FGF-2-heparin binary and FGF-2-heparin-FGFR1 ternary complex interactions and to have these interactions inhibited by antiangiogenic but not native forms of antithrombin. The inability of native antithrombin to affect these growth factor-heparin interactions therefore does not involve the sequestering of native antithrombin by pentasaccharide sequences in unfractionated heparin and affirms that native and antiangiogenic forms of antithrombin make distinct types of interactions with heparin. Similarly, native antithrombin did not affect FGF-2 binding to its low affinity heparan sulfate co-receptor on endothelial cells or signaling that is dependent on FGF-2 binding to its endothelial cell receptor, i.e. FGF receptor-1 autophosphorylation, p42/44 MAP kinase phosphorylation, and HUVEC proliferation. The heparan sulfate-dependent stimulation of HUVEC proliferation by VEGF, which was inhibitable by antiangiogenic forms of antithrombin, was additionally not affected by native antithrombin. Antiangiogenic forms of antithrombin thus appear to recognize heparan sulfate domains that overlap or coincide with the specific domains that mediate growth factor-receptor-heparan sulfate ternary complex for-
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