ANTI-ANGIOGENIC ANTITHROMBIN DOWNREGULATES THE EXPRESSION OF THE PRO-ANGIOGENIC HEPARAN SULFATE PROTEOGLYCAN, PERLECAN, IN ENDOTHELIAL CELLS*

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ABBREVIATIONS

bFGF, basic fibroblast growth factor; EST, expressed sequence tag; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVECs, human umbilical vein endothelial cells; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT, reverse transcriptase; SDS, sodium dodecyl sulfate; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor
SUMMARY

Antithrombin, a key serpin family regulator of blood coagulation proteases, is transformed into a potent anti-angiogenic factor by limited proteolysis or mild heating. Here, we show by cDNA microarray, semiquantitative RT-PCR, Northern blotting and immunoblotting analyses that the expression of the pro-angiogenic heparan sulfate proteoglycan (HSPG), perlecan, but not other HSPGs, is dramatically downregulated in human umbilical vein endothelial cells (HUVECs) treated with anti-angiogenic cleaved and latent forms of antithrombin, but not with the native form. Downregulation of perlecan expression by cleaved and latent antithrombins was observed in both basic fibroblast growth factor (bFGF)-stimulated and unstimulated cells, whereas the anti-angiogenic antithrombins inhibited the proliferation of only bFGF-stimulated HUVECs by arresting cells at the G1 cell cycle phase. The importance of perlecan expression levels in mediating the anti-proliferative effect of the anti-angiogenic antithrombins was suggested by the finding that transforming growth factor-β1, a potent stimulator of perlecan expression in endothelial cells, blocked the downregulation of perlecan expression and anti-proliferative activity of cleaved antithrombin on endothelial cells. The previously established key role of perlecan in mediating bFGF stimulation of endothelial cell proliferation and angiogenesis suggests that a primary mechanism by which anti-angiogenic antithrombins exert their effects is through the downregulation of perlecan expression.
INTRODUCTION

Antithrombin, a member of the serpin superfamily of proteins, is an essential anticoagulant regulator of blood coagulation.\textsuperscript{1,2} This regulation is achieved principally through the inhibition of the clotting cascade proteases, factor IXa, factor Xa and thrombin, and requires the glycosaminoglycan, heparin, as a cofactor. Heparin binds antithrombin with high affinity and thereby activates the serpin to inhibit clotting proteases at a physiologically significant rate.\textsuperscript{3,4} In addition to its role in hemostasis, antithrombin has more recently been shown to possess a variety of other biologic activities which are anti-inflammatory, anti-viral and anti-angiogenic.\textsuperscript{5-7} The anti-angiogenic activity is produced only by conformationally altered forms of antithrombin produced by proteolytic cleavage or transition to a latent or prelatent form and is evidenced by the ability of these antithrombin forms to inhibit blood vessel growth in the chick embryo, to inhibit the proliferation of endothelial cells in culture and to induce tumor regression in mice.\textsuperscript{7-9} The physiologic relevance of this activity is suggested by the finding that primary human pancreatic cancer cells can inhibit secondary tumor growth in mice due to their ability to convert endogenous native antithrombin into the anti-angiogenic cleaved and latent forms of the serpin.\textsuperscript{10}

As a member of the serpin superfamily, antithrombin shares a common tertiary structure with other serpins and functions as protease inhibitor like other inhibitory members of the family.\textsuperscript{2} The conformational changes in antithrombin that result in the cleaved and latent anti-angiogenic forms are similar, both involving the insertion of an exposed reactive loop of the serpin into the major $\beta$-sheet of the protein core, the A sheet.\textsuperscript{11,12} In the case of the latent form, the insertion of the loop is induced by mild heating without proteolysis\textsuperscript{13} whereas for the cleaved form, limited proteolysis in the loop is sufficient to cause the insertion.\textsuperscript{14,15} A similar cleavage-
induced insertion of the loop into sheet A occurs when the serpin forms a stable complex with a target protease and this is associated with a large-scale movement and deformation of the protease.\textsuperscript{16,17} However, reactive loop insertion does not appear to be a requirement for the induction of anti-angiogenic activity in antithrombin since a conformationally altered form in which this insertion had not occurred was found to produce anti-angiogenic activity comparable to that of the cleaved and latent antithrombins.\textsuperscript{9} Both cleaved and latent forms of antithrombin are produced under normal physiologic conditions,\textsuperscript{18,19} suggesting that their anti-angiogenic activity may be physiologically relevant.

While the anti-angiogenic activity of conformationally altered forms of antithrombin appears to be well established, the molecular mechanisms underlying the serpin's anti-angiogenic activity remain to be elucidated. Since angiogenesis is a complex physiological process thought to be controlled by multiple genes,\textsuperscript{20} we expected that anti-angiogenic forms of antithrombin would induce their anti-proliferative effect on endothelial cells by altering the profile of expressed genes. In the present study, the effects of cleaved and latent anti-angiogenic forms of antithrombin and of native antithrombin on the expression of genes in primary human umbilical vein endothelial cells (HUVECs) were analyzed by the microarray technique.\textsuperscript{21} A striking finding was that the anti-angiogenic antithrombins but not the native serpin produced a dramatic downregulation of the gene for the pro-angiogenic heparan sulfate proteoglycan, perlecan.\textsuperscript{22,23} This downregulation was confirmed at the mRNA level by Northern blotting and semiquantitative RT-PCR and at the protein level by immunoblotting and was correlated with the inhibition of bFGF-stimulated endothelial cell proliferation and the blocking of the G1-S phase transition in cultured HUVECs by the anti-angiogenic antithrombins. Up-regulation of perlecan
gene expression in HUVECs by TGF-β1 overcame the inhibition of HUVEC proliferation produced by the anti-angiogenic antithrombins. Collectively, our results demonstrate a strong correlation between antithrombin anti-angiogenic activity and the down-regulation of perlecan gene expression.

MATERIALS AND METHODS

Materials - Oligonucleotides for PCR analysis were synthesized by Sigma-Aldrich (St. Louis, MO). TGF-β1 was purchased from Oncogene (San Diego, CA) and bFGF was from Invitrogen (Carlsbad, CA). Human umbilical vein endothelial cells (HUVECs) were obtained either from the American Type Culture Collection (Manassas, VA) or isolated from freshly harvested umbilical cords from the Stanford University Medical Center as described below. A mouse monoclonal antibody to human perlecan and anti-mouse IgG labeled with horseradish peroxidase were purchased from ZYMED (San Francisco, CA). Neutrophil elastase was purchased from Athens Research and Technology (Athens, GA) and human thrombin was a gift of John Fenton (NY Department of Public Health, Albany, NY).

Preparation and purification of native and conformationally altered forms of human plasma antithrombin- Antithrombin was purified from human plasma as described previously. The cleaved form of antithrombin was generated either by human thrombin cleavage in the presence of heparin in a low ionic strength buffer or by neutrophil elastase cleavage as described. The latent form of antithrombin was prepared by incubating the serpin at 50-60 °C in the presence of sodium citrate as described. Cleaved and latent forms of antithrombin were purified by Hi-Trap Heparin and Mono-Q chromatography (Amersham Biosciences, Piscataway, NJ) as in previous studies. SDS and native PAGE confirmed that the cleaved and latent forms had the expected
changes in electrophoretic mobility when compared with the native serpin. Both forms lacked detectable inhibitor activity against thrombin.

Isolation of Primary HUVECs: Fresh umbilical cords were obtained 2-6 h after normal term deliveries from Dr. Maurice L. Druzin of the Stanford University Medical Center, Department of Gynecology and Obstetrics. Human umbilical vein endothelial cells were isolated as previously published with slight modification. Briefly, the umbilical vein was cannulated with a luer adapter and perfused with collagenase (1000U/ml, Type-2, Worthington Biochemical Corp., Lakewood, NJ) at 37°C for 10 min. Cells liberated by the collagenase treatment were collected in EGM-MV EC medium (Cambrex Bioscience, Walkersville, MD) and cultured in 0.2% gelatin-coated Falcon T25 flask. For those HUVECs containing significant contaminating red blood cells, the cells were centrifuged in a 35% Percoll gradient (Amersham Biosciences) to remove the red cells.

Microarray Analysis: Confluent passage-2 HUVECs in Falcon T12.5 flasks grown in the presence or absence of VEGF-165 (10 ng/mL) (Sigma-Aldrich) were treated with native, cleaved or latent antithrombins (20 µg/mL) for 24 hours in 3 mL M-199 with 2% heat-inactivated FBS and antibiotics. Following this treatment, total RNA was extracted from the cells by the Trizol method (Invitrogen). The total RNA was subjected to one cycle of linear RNA amplification using the MessageAMp aRNA kit according to the manufacturer’s instructions (Ambion, Austin, TX), which routinely generated ~7-10 µg antisense RNA for every microgram of total RNA extracted from confluent HUVEC cultured in a T12.5 flask. The quality of the antisense RNA was verified on a denaturing agarose gel. Only samples showing a distribution of sizes from 250-5500 nt with a peak centered at 1000-1500 nt were used to generate the “test” cDNA samples for
the subsequent array experiments. DNA labeling and hybridizations were performed essentially as described,\textsuperscript{21} with slight modifications. Briefly, the “test” endothelial cell antisense RNA samples together with Universal Human Reference RNA (Stratagene, Cedar Creek, TX) were used to generate Cy3/Cy5 (Amersham Biosciences) labeled cDNA for array hybridization on the Stanford 43K human cDNA microarray (http://www.microarray.org/sfgf/jsp/servicesFrame.jsp#productionArrays). The Stanford microarray used in this study consists of 18,416 named genes with UniGene symbol, 4,145 ESTs with known function, 19,365 ESTs with unknown function and ~1,000 repeated spots as internal controls. Hybridized arrays were scanned on a GenePix 4000B scanner (Axon Instruments), and fluorescence ratios (test/reference) calculated using the Stanford Microarray Database software (available at http://genome-www.stanford.edu/microarray).\textsuperscript{29} Fluorescence ratios were normalized for each array by setting the average log fluorescence ratio for all array elements equal to 0. Genes whose expression was at least 3-fold induced or 3-fold repressed by growth factor or antithrombin treatment in at least 1 experiment with regression correlation more than 0.6 were considered significant. For UniGene clusters represented by multiple arrayed elements, mean fluorescence ratios (for all elements representing the same UniGene cluster) are reported. The entire data set described here can be accessed at the Stanford Microarray Database.

\textit{Cell culture and Cell Proliferation Assays}- Commercially procured HUVECs were routinely maintained in F12-K medium with 10\% FBS, 100 \( \mu \)g/ml heparin, 30-50 \( \mu \)g/ml endothelial cell growth supplement and 1\% penicillin and streptomycin (Invitrogen). To determine the growth rates of cells exposed to various treatments, only the cells between passage 5 and 10 were used. 5000-6000/well HUVECs were seeded into 96 well plates in triplicate. After attachment, cells
were made quiescent by incubation with F12-K medium containing 0.2% FBS plus 1% penicillin and streptomycin. After 24 hours incubation, fresh medium containing various combinations of additional agents including native, cleaved or latent forms of antithrombin (10-20μg/ml), TGF-β1 (5ng/ml) or bFGF (10ng/ml) was added and the cells incubated for another 48 hours. The number of viable cells was quantitated using the non-radioactive colorimetric CellTiter 96 AQ Cell Proliferation Assay according to the manufacturer's instructions (Promega, Madison, WI).

Cell Cycle Analysis- The effects of bFGF and antithrombin treatments on the cell cycle distribution of cultured HUVECs was analyzed according to Hanai et al. HUVECs were growth-arrested by contact inhibition for 48 h. The cells (0.1 × 10^6 cells/well) were harvested and plated onto a T25 flask in duplicate in endothelial cell growth medium containing 2% FBS and either native, cleaved or latent forms of antithrombin (20μg/ml) in the presence and absence of recombinant bFGF (10 ng/ml). The cells were harvested after 72-hour and then fixed in ice-cold 70% ethanol. Fixed cells were incubated at 4 °C for 30 min in phosphate-buffered saline containing 2% FBS and 0.1% Tween 20, and then centrifuged and resuspended in 0.5 ml of the same buffer. RNase digestion (5 μg/ml) was carried out at 37 °C for 1 h followed by staining with propidium iodide (5 μg/ml). The cells in G1, S and G2 phases were quantitated using a FACScan BD PharMingen flow cytometer and Multicycle AV software (Phoenix Flow Systems, San Diego, CA).

Isolation of RNA and RT-PCR Analysis—Total mRNA from HUVECs was isolated using the RT-PCR Miniprep kit (Stratgene) according to the manufacturer's instructions. Semi-quantitative RT-PCR assays were performed by synthesizing a first strand cDNA from 200 ng of total mRNA with Stratascript RT (Stratagene). PCR amplification of perlecan cDNA was then performed.
using primers from perlecan domain III,\textsuperscript{31} 5'-ACAGTGCAACAAGTGCAAGG-3' and 5'-CTGAAGTGACCAGGCTCCTC-3', which were expected to amplify a 500bp perlecan gene fragment (nt. +2450 - +2950). Simultaneous amplification of human cytoplasmic β-actin\textsuperscript{25} as a reference used the primers, 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC- 3’ and 5'-ATCTGGCACCACACCTTCTACAAATGAGCTGCG-3’, which were expected to amplify a 336 bp cDNA fragment. Semi-quantitative PCR reactions were performed using the QIAGEN PCR Kit (Qiagen Sciences, Germanstown, MD) and included 0.1 µM each of the two β-actin primers, 0.16 µM each of the perlecan gene-specific primers and ~5 ng cDNA template. 26 PCR cycles were found to yield amounts of perlecan and β-actin products that were in the linear range of the logarithm of the product vs. number of PCR cycles. Reactions containing all PCR components except for the cDNA template were also amplified as a negative control to check for the presence of contaminating DNA. The products of PCR reactions were separated by electrophoresis on 1.5% agarose gels and detected by staining with Sybr Green dye (Molecular Probes, Eugene OR). The intensity of PCR product bands was quantitated with a Kodak Image Station 440CF (Eastman Kodak, Rochester, NY).

*Northern Blot Analysis*- Isolation of total RNA from HUVECs was performed as above. The RNA was formaldehyde-denatured, fractionated on 1.2% agarose gels, transferred by capillary blotting onto Hybond N membranes (Amersham Buchler, Braunschweig, Germany), and fixed by UV-cross-linking. The Northern blots were probed with a P\textsuperscript{32}-labeled 500-bp perlecan cDNA synthesized by RT-PCR as described above and with a labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a loading control. Blots were exposed to X-ray film for detection of bound probe and band intensities quantitated with the Kodak Image Station.
Slot Immunoblotting - For immunoblotting, $10^6$ cells were seeded in six-well dishes and incubated in serum-free medium. After 12 hours incubation, cells were replaced with fresh serum-free medium and various treatment agents added as above and the cells were then incubated for 72 hours. Serial amounts of the medium were applied to nitrocellulose filters (Schleicher and Schuell, Keene, NH) and air-dried. The membranes were blocked with 5% Carnation non-fat dry milk in Tris-borate buffer containing 0.1% NP-40 and incubated with a monoclonal antibody directed against domain III of perlecan for one hour at room temperature. After three washes with blocking buffer, the membranes were incubated with an anti-mouse IgG labeled with horseradish peroxidase for another hour at room temperature, washed with buffer, and then bound antibody was detected by incubation with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) followed by autoradiography for 10-60 s. Band intensities were quantified with the Kodak Image Station.

RESULTS

Anti-proliferative effects of antithrombin forms- The effects of native, cleaved and latent antithrombin forms on the proliferation of cultured HUVECs was analyzed in the presence and absence of bFGF (Fig. 1). bFGF stimulated the growth of resting HUVECs up to 180% and latent and cleaved antithrombins significantly inhibited this stimulation whereas native antithrombin had no effect. In contrast, the basal growth rate of HUVECs in the absence of bFGF was not significantly influenced by treatment with any of the three antithrombin forms. Varying the dosage of the cleaved and latent antithrombins used to treat bFGF-stimulated cells showed that the concentration employed in the experiments of fig. 1 (10 µg/ml) was sufficient for maximal inhibition of cell growth (not shown). No significant differences were found between cleaved
antithrombins prepared by thrombin or neutrophil elastase cleavage in this assay. Similar
differential effects of the antithrombins on the growth of primary HUVECs were observed when
VEGF was used to stimulate the cells (not shown). These results confirm the anti-proliferative
effects of the latent and cleaved forms of antithrombin and inability of native antithrombin to
produce such effects as has been established in previous studies.7,8

Effects on G1-S phase transition—Since growth factors such as bFGF induce cells to exit from a
quiescent phase (G0/G1) into an active dividing phase (S phase)30 and cleaved and latent forms of
antithrombin inhibit growth factor-induced cell proliferation, it was of interest to determine the
effects of the three forms of antithrombin on the transition from the G1 to the S phase in
HUVECs stimulated by bFGF. Flow cytometric analyses based on the DNA content of HUVECs
treated with the three forms of antithrombin in the presence and absence of bFGF showed the
distribution of cells at each phase of the cell cycle for each of the conditions tested (Fig. 2). bFGF
increased the fraction of cells making the transition from the resting phase (G1) to the DNA
synthesis phase (S) by ~3-fold (from 5.5% to 14%) and this enhancement was unaffected by
treatment with native antithrombin. However, treatment of the bFGF-stimulated cells with latent
or cleaved antithrombin abolished the enhancement in the fraction of cells in S phase. No effects
on the fraction of cells in S phase were observed in those HUVECs treated with any of the three
forms of antithrombin in the absence of bFGF (data not shown). These results showed that
cleaved and latent forms of antithrombin inhibit the G1-S phase cell cycle transition in growth
factor-stimulated HUVECs, but not in unstimulated quiescent cells.

cDNA Microarray profiling of changes in endothelial cell gene expression - To investigate the
mechanism by which latent and cleaved forms of antithrombin inhibit the bFGF-stimulated
proliferation of endothelial cells, we analyzed the transcript profile of primary HUVECs untreated and treated with the three forms of antithrombin in the presence and absence of vascular endothelial growth factor (VEGF) using the Stanford 43K human cDNA microarray. The gene expression patterns of HUVECs incubated for 24 hours with the three forms of antithrombin in the presence and absence of VEGF were compared with untreated controls. Of particular note was the effect of the antithrombins on the expression of the gene for the heparan sulfate proteoglycan, perlecan (fig. 3). Perlecan gene expression was significantly suppressed from 3-6-fold in cells treated with latent antithrombin and >10-fold in cells treated with cleaved antithrombin relative to its expression in untreated cells or cells treated with native antithrombin and this suppressive effect was observed whether cells were cultured with or without VEGF. By contrast, the expression of other heparan sulfate proteoglycans including syndecan, glypican, betaglycan and CD44 was 10-20-fold less than that of perlecan with or without VEGF and not greatly affected by the antithrombin forms (fig. 3).

RT-PCR, Northern blotting and immunoblotting analyses of perlecan gene expression-Because of the key role of perlecan in angiogenesis, tissue remodeling and cell transformation, we wished to confirm that perlecan gene expression was suppressed in HUVECs treated with anti-angiogenic forms of antithrombin. Relative perlecan mRNA levels were therefore analyzed by semiquantitative RT-PCR in cultured HUVECs treated with the different forms of antithrombin with or without bFGF after establishing the linear range of the assay and normalizing the results to an internal β-actin control (fig. 4). HUVECs treated with bFGF contained 1.8 times more perlecan mRNA than untreated cells and when native antithrombin was present, bFGF caused perlecan mRNA to increase an even greater 2.7-fold in the cells. In contrast, perlecan mRNA
levels in cells treated with latent or cleaved forms of antithrombin were all reduced from the untreated cells by 2-3-fold whether cells were cultured in the presence or absence of bFGF. These results confirmed the findings of the cDNA microarray analysis, i.e., that anti-angiogenic forms of antithrombin not only abolished bFGF-enhanced expression of the perlecan gene in HUVECs, but also suppressed the basal level of perlecan gene expression.

To further confirm the down-regulation of perlecan gene expression in endothelial cells treated with anti-angiogenic forms of antithrombin, total RNAs from HUVECs were isolated and perlecan mRNA levels were quantitated by northern blot analysis after normalization of results to an internal control (Fig.5). Perlecan mRNA levels were increased ~4-fold in cells cultured in the presence of bFGF relative to the levels in cells cultured without bFGF when native antithrombin was present. However, the bFGF-mediated enhancements of perlecan mRNA levels were completely inhibited in HUVECs cultured in the presence of latent or cleaved forms of antithrombin. Although the levels of perlecan mRNA in unstimulated cells also appeared to be lower in cells treated with the anti-angiogenic antithrombins than with native antithrombin, the differences could not be reliably determined at the low expression levels observed. These results nevertheless confirmed the marked downregulation of perlecan transcript in bFGF-stimulated cells treated with the anti-angiogenic antithrombins.

To determine if the down-regulation of perlecan mRNA levels by anti-angiogenic forms of antithrombin was paralleled by a decrease in perlecan protein levels, we analyzed perlecan protein in the medium of cultured cells by immunoblotting using a perlecan domain III-specific antibody (fig. 6). Perlecan protein levels were significantly increased ~2-3-fold in cells cultured in the presence of bFGF plus native antithrombin as compared to cells cultured in the absence of growth factor. The bFGF-enhancement of perlecan protein levels was completely abolished in
cells treated with latent or cleaved forms of antithrombin and perlecan levels were in fact reduced from the level found in unstimulated cells. Treatment of HUVECs cultured in the absence of bFGF with latent and cleaved forms of antithrombin also appeared to reduce the levels of perlecan protein from those observed in cells treated with native antithrombin. Together, these results established that anti-angiogenic forms of antithrombin significantly down-regulate perlecan gene expression in growth factor-stimulated and unstimulated HUVECs.

Modulation of antithrombin anti-proliferative activity by TGF-β1- Since bFGF and anti-angiogenic forms of antithrombin exert opposite effects on cell proliferation and perlecan gene expression in HUVECs, we evaluated how the anti-angiogenic activity of latent and cleaved antithrombins would be affected by another known stimulator of perlecan gene expression, TGF-β1 (Fig. 7). Control experiments verified that bFGF stimulated HUVEC proliferation and this stimulation was not affected by treatment with native antithrombin but was nearly abolished in HUVECs treated with cleaved antithrombin. TGF-β1 did not significantly affect the growth of quiescent HUVECs in the absence or presence of native antithrombin. However, TGF-β1 amplified the stimulation by bFGF of HUVEC proliferation and this enhanced proliferation was only slightly inhibited by cleaved antithrombin (Fig. 7A). Relative perlecan mRNA levels measured by semi-quantitative RT PCR were significantly increased in bFGF-stimulated HUVECs treated with TGF-β1 when compared with non-treated cells and additional treatment with cleaved antithrombin did not significantly affect the mRNA levels (Fig. 7B). These results indicate that over-expression of the perlecan gene overcomes the anti-proliferative effect of cleaved and latent antithrombins on HUVECs.

DISCUSSION
The results of the present study have shown that the anti-proliferative effects of the cleaved and latent conformations of antithrombin on human umbilical vein endothelial cells, a component of the more complex anti-angiogenic biologic activity of these antithrombin forms, is correlated with the downregulation of a key pro-angiogenic factor, the extracellular matrix heparan sulfate proteoglycan, perlecan. Downregulation of perlecan expression was shown to occur both at the mRNA and protein levels, to be induced by the anti-angiogenic cleaved and latent conformations of antithrombin and not the native serpin conformation, and to be dramatic, i.e., up to ~6-10-fold by microarray analysis of primary low passage HUVEC cultures and ~3-4-fold by semi-quantitative RT-PCR and northern blot analyses of somewhat higher passage HUVEC cultures. Perlecan downregulation was observed in both resting and growth factor-stimulated endothelial cells. The expression of other endothelial cell heparan sulfate proteoglycans were not greatly affected by any of the antithrombin forms, indicating that the anti-angiogenic antithrombins specifically affected perlecan-type heparan sulfate proteoglycan expression in HUVECs.

We confirmed the observations of past studies that anti-angiogenic forms of antithrombin principally inhibit the proliferation of growth factor-stimulated endothelial cells and minimally affect the growth of unstimulated cells, although our studies used HUVECs instead of the porcine aortic or bovine capillary endothelial cells used in past studies. The inhibition of bFGF-stimulated HUVEC proliferation by cleaved and latent antithrombins was shown to arise through the suppression of the growth factor-enhanced transition of cells from the G1 to the S phase of the cell cycle. The anti-angiogenic collagen XVIII fragment, endostatin, produces an anti-proliferative effect on endothelial cells by a similar mechanism. That perlecan levels critically mediate the growth factor-dependent proliferation of endothelial cells was shown by the
finding that upregulation of perlecan expression by TGFβ1 overcame the anti-proliferative effects of the anti-angiogenic antithrombins on HUVECs. The similar growth factor-dependent anti-proliferative activity of cleaved and latent antithrombins on aortic, capillary and umbilical vein endothelial cells observed in the present and past studies suggests that perlecan downregulation may be a common mediator of this activity, although further studies will be required to validate this possibility.

The dramatic effects of anti-angiogenic antithrombins on perlecan expression provide a readily understandable mechanism by which these antithrombins may block growth factor-stimulated endothelial cell proliferation as well as inhibit other reported growth factor-dependent activities associated with angiogenesis. Perlecan is a major heparan sulfate proteoglycan secreted by endothelial cells and highly expressed in several tumor cell lines. It functions as an essential co-receptor for bFGF and VEGF family growth factors, enabling them to bind and activate their receptors and to thereby promote cell growth and differentiation. In particular, perlecan-bFGF complexes mediate the pro-angiogenic activity of bFGF in which endothelial cells are stimulated to form new blood vessels. The co-receptor function of perlecan results from bFGF binding to the heparan sulfate chains of the proteoglycan. Suppression of perlecan expression by antisense approaches blocks bFGF activity and proliferation in 3T3 fibroblasts and several tumor cell lines as well as tumor cell-induced angiogenesis and these activities are restored by addition of exogenous perlecan. A mouse knockout of the perlecan gene causes aberrant cartilage development thought to reflect defective growth factor signalling. The well established inhibition of bFGF-stimulated endothelial cell growth and angiogenesis as well as angiogenesis-dependent tumor growth by cleaved and latent forms of antithrombin could thus be due to the decreased levels of perlecan heparan sulfate chains and consequent inability of the
growth factors to bind and activate their receptors. Other growth factor-dependent effects involved in angiogenesis and inhibited by anti-angiogenic antithrombins include focal adhesion kinase activation, focal adhesion contact formation and actin reorganization. All of these effects may similarly be accounted for by the blocking of growth factor stimulation of cells by anti-angiogenic antithrombins through the downregulation of perlecan expression.

Our finding that perlecan expression is downregulated also in unstimulated endothelial cells without any significant effects on the growth or distribution of cells in G1 and S cell cycle phases suggests that perlecan levels are not critical for the growth of resting cells. Similarly, in 3T3 fibroblast and melanoma cells, suppression of perlecan gene expression by an anti-sense cDNA only affected the response of cells to bFGF stimulated growth and not the basal growth rate. Anti-angiogenic forms of antithrombin have also been shown to enhance apoptosis in both resting and growth factor-stimulated endothelial cells. The observation of both growth factor-independent and dependent effects of cleaved and latent but not native forms of antithrombin on endothelial cells suggests that the anti-angiogenic antithrombins produce global effects on endothelial cells independent of growth factor binding presumably mediated by a specific endothelial cell receptor interaction. Binding of the anti-angiogenic antithrombins to this putative receptor presumably blocks growth factor-dependent angiogenesis activity by downregulating perlecan expression and stimulating apoptosis.

Endostatin acts as an inhibitor of bFGF-dependent angiogenesis, but the anti-angiogenic activity appears to be due in this case to endostatin binding to heparan sulfate proteoglycan coreceptors which thereby blocks growth factor binding and activation of endothelial cells. This mechanism is suggested by the demonstrated requirement of the heparin binding site of endostatin for this activity and identification of specific heparan sulfate binding domains which
mediate the activity. While our results suggest that anti-angiogenic forms of antithrombin bind to an endothelial cell receptor to produce their effects, it is possible that these antithrombins may also require a heparan sulfate coreceptor to mediate this binding as is the case with bFGF and VEGF binding to their receptors. Cleaved and latent anti-angiogenic forms of antithrombin bind heparin, but have greatly reduced affinity for the polysaccharide when compared to the nonanti-angiogenic native antithrombin conformation. The higher affinity of native antithrombin for heparin is due to the recognition of a specific anticoagulant sequence present in a small fraction of the chains. Binding of anti-angiogenic antithrombins to non-anticoagulant heparan sulfate sequences could thus play a role in mediating the anti-angiogenic effects of the serpin. This would be in keeping with the observation that anticoagulant heparan sulfate sequences associated with endothelial cells are localized in membrane-associated glypican-type heparan sulfate proteoglycans whereas nonanticoagulant chains which bind antithrombin exist on matrix-associated perlecan. Future studies will need to address the importance of the heparin binding site of antithrombin for its anti-angiogenic activity.

In summary, our results have shown that the anti-proliferative effects of cleaved and latent forms of antithrombin on endothelial cells and presumably also the anti-angiogenic effects may be explained by these antithrombins inducing a downregulation of perlecan expression. Since perlecan is an essential co-receptor for bFGF-induced angiogenesis, the downregulation of this coreceptor in endothelial cells by the anti-angiogenic antithrombins can explain why bFGF-dependent angiogenesis is inhibited. This proposal is supported by the similar inhibition of bFGF-dependent proliferation and angiogenesis in tumor cells produced by downregulation of perlecan expression using antisense methods. Moreover, the inhibition of bFGF-dependent endothelial cell proliferation by anti-angiogenic antithrombins can be overcome through the
upregulation of perlecan expression by TGFβ1.\textsuperscript{34} Perlecan expression thus appears to be a key means of regulating the activities of both pro- and anti-angiogenic factors in endothelial cells and possibly in tumor cells as well.

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REFERENCES


FIGURE LEGENDS

Figure 1. Inhibition of bFGF-induced endothelial cell proliferation by cleaved and latent antithrombins- Resting HUVEC cells were cultured in the absence or presence of 10µg/ml each of native, cleaved or latent forms of antithrombin both in the absence and presence of 10ng/ml of bFGF as indicated. The number of viable cells was determined after 72 hours incubation as described in "Materials and Methods". Mean values ± S.D. (bars) derived from five independent determinations are shown. * indicates a statistically significant difference from the control (p<0.001) based on a student t-test.

Figure 2. Effects of antithrombin forms on endothelial cell cycle transitions- Synchronized HUVEC cells (2×10⁶) were cultured with different antithrombin forms (20µg/ml) in the presence or absence of bFGF (10ng/ml) for 48 hours. Cells were fixed and stained with propidium iodide for detection of DNA and then analyzed by flow cytometry as detailed in "Materials and Methods". Panel A shows plots of the distribution of cells among the G1, S, and G2 phases of the cell cycle as reflected by their DNA content under the indicated culture conditions Panel B shows the percentage of cells in each cell cycle phase measured from the data in panel A and other experiments as mean values ± S.D. (bars) from three independent determinations. The different bar patterns represent from left to right the same sequence of conditions given in panel A. * indicates a statistically significant difference from the control ( p <0.001).

Figure 3 cDNA microarray analysis of the effects of antithrombin forms on the expression of select heparan sulfate proteoglycans in primary HUVECs HUVECs were cultured with or without different antithrombin forms (20µg/ml) in the presence and absence of VEGF (10ng/ml) as indicated. Total mRNA was isolated from the cells, amplified and analyzed by cDNA
microarray as detailed in "Materials and Methods". The expression of the indicated heparan sulfate proteoglycan mRNAs relative to a universal RNA reference is shown for the different treatment conditions ± S.E.M.

Figure 4. **Semi-quantitative RT-PCR analysis of perlecan mRNA expression in antithrombin-treated HUVECs**

HUVECs were cultured with or without 10 µg/ml of the different antithrombin forms in the presence and absence of 10 ng/ml bFGF as indicated for 72 hours. Total mRNA was isolated and the content of perlecan mRNA relative to β-actin mRNA was analyzed by semi-quantitative RT-PCR. The inset shows the relative intensities of 500 bp perlecan and 336 bp β-actin cDNA fragments amplified from reverse transcribed mRNA with specific primers under the different experimental conditions, as described in "Materials and Methods". Band intensities from the representative experiment shown were quantified and are presented as the ratio of perlecan to β-actin bands in the bar graph. Similar results were obtained in replicate experiments.

Figure 5. **Northern blotting analysis of perlecan mRNA expression in antithrombin-treated HUVECs**

Total mRNA was isolated from HUVECs cultured under the conditions of fig. 4 and perlecan mRNA expression analyzed by northern blotting using a perlecan domain III cDNA probe as described in "Materials and Methods". The same blot was probed with GAPDH cDNA as a loading control. Normalized band intensities are shown.

Figure 6. **Cleaved and latent forms of antithrombin downregulate perlecan protein expression**

HUVECs were cultured in serum-free media with or without 10 µg/ml of the different forms of antithrombin in the presence and absence of 10 ng/ml bFGF as indicated for 72 hours. The conditioned media was applied to a nitrocellulose membrane in the indicated amounts
and perlecan protein was detected by enhanced chemiluminescence using a primary antibody to
domain III of perlecan followed by a secondary enzyme-conjugated antibody as detailed in
"Materials and Methods".

Figure 7. Reversal of the anti-proliferative effect of cleaved antithrombin on bFGF-
stimulated HUVECs by TGF-β1-induced over-expression of perlecan HUVECs were
cultured with or without 10 µg/ml of native (N) or cleaved (C) forms of antithrombin in the
absence or presence of 10ng/ml bFGF and/or 5 ng/ml TGF-β1 as indicated for 48 hours and then
the number of viable cells were counted as described in "Materials and Methods". Panel A shows
the number of viable cells expressed relative to the control as mean ± S.D. (bars) from three
independent determinations. Panel B shows the relative perlecan and β-actin control mRNA
levels measured in bFGF-stimulated HUVECs cultured in the absence (Lane 1-3) or in the
presence of TGF-β1 (Lane 4-6) by semi-quantitative RT PCR as in figure 4. * in panel A
indicates a statistically significant difference from the control in the absence of TGF-β1( p
<0.001).
Figure 1
Figure 2
Figure 3

Relative Expression (Test/Reference)

Resting
+VEGF
+Native
+VEGF/Native
+Latent
+VEGF/Latent
+Cleaved
+VEGF/Cleaved

Perlecan  CD44  Syndecan 2  Betaglycan  Glypican 1
Figure 4

Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8
-----|---|---|---|---|---|---|---|---
AT   | None | Native | Latent | Cleaved
bFGF | - | + | - | + | - | + | - | +

Relative Perlecan mRNA levels

Perlecan
β-actin
Figure 5
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**Figure 6**
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**Figure 7B**
Anti-angiogenic antithrombin downregulates the expression of the pro-angiogenic heparan sulfate proteoglycan, perlecan, in endothelial cells

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