Endometrial Angiopoietin Expression and Modulation by Thrombin and Steroid Hormones

A Mechanism for Abnormal Angiogenesis Following Long-Term Progestin-Only Contraception

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The angiopoietins (Ang) are endothelial cell-related factors necessary for the development and maintenance of all vessels. Altering the expression of these proteins would be expected to result in aberrant angiogenesis. Indeed the fragile endometrial vasculature and bleeding observed in women treated with long-term progestin-only contraceptives has been associated with changes in the expression of Ang-1 and Ang-2. Since bleeding would result in thrombin formation, we have assessed the effects of thrombin on the expression of the Angs in human endometrial cells. This study shows that thrombin significantly reduces the expression of Ang-1 protein and mRNA expression in human endometrial stromal cells (HESCs) and minimally decreases the production of Ang-2 protein in human endometrial endothelial cells (HEECs). Hence the presence of thrombin due to aberrant bleeding could affect the angiogenic potential of the endometrium, creating a feed forward loop resulting in more thrombin, weak vasculature, and more bleeding. In addition, since the exact localization of Ang in the human endometrium remains a subject of controversy, we have addressed this issue in an *in vivo* system by analyzing the expression of Angs by microdissection of HESCs, HEECs, and human endometrial glandular epithelial cells followed by real time, quantitative RT-PCR. (Am J Pathol 2004, 164:2101–2107)

Thrombin stimulation has been shown to induce vascular permeability and endothelial cell migration and proliferation.^{1–3} Many of thrombin's effects are now known to be mediated by members of the protease-activated receptor (PAR) family.⁴ Vascular injury results in local thrombin

generation and angiogenesis. Hence these two mechanisms are intimately associated and likely regulated in a coordinate fashion.

Two protein families, namely vascular endothelial growth factor (VEGF) and the angiopoietins (Angs), are the major effectors of angiogenesis. Thus, a putative role for thrombin's involvement in angiogenesis is likely to involve the regulation of these proteins. Indeed thrombin up-regulates the expression of VEGF receptors in endothelial cells and has also been shown to induce the expression of VEGF in cancer cells⁵ and in decidualized human endometrial stromal cells (HESCs).⁶ Recently, thrombin has been shown to induce the expression of Ang-2 in human umbilical vein endothelial cells.⁷

The human endometrium undergoes classical cyclical changes culminating in menstruation, the latter necessitates renewal and an increased demand for angiogenesis.^{8–10} However, pathological endometrial bleeding such as that observed following long-term progestin-only contraception or hormone replacement therapy is likely to result in sustained focal thrombin production. To date, the effect of thrombin on the expression of both Ang-1 and Ang-2 by the human endometrium has not been studied. Since these are critical molecules both in vascular remodeling and maintenance, aberrant expression of these cells could result in vessel fragility and bleeding. This study examines the effects of thrombin on the expression of Ang-1 and Ang-2 by the endometrium.

Materials and Methods

Human Endometrial Cell Isolation and Cell Culture

Endometria from reproductive age women were obtained from hysterectomies for benign conditions (eg, myomas) after obtaining written informed consent and institutional

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approval from the Human Investigation Committee of Yale New Haven Hospital. The endometria were transported to a sterile laminar flow hood and isolation of the cells was conducted as follows. Primary human endometrial stromal cells (HESCs) were obtained and grown to confluence and incubated in parallel for 7 days in 0.1% ethanol (vehicle control) or 10^{-8} M E_2 or 10^{-7} M medroxyprogesterone acetate (MPA) (Sigma-Aldrich, St. Louis, MO) as previously described.¹¹ Briefly, endometrium was scraped out and placed in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) at 4°C, the tissue was transferred to Hanks' balanced salt solution. After cleaning, the tissue was finely minced and spun for 10 minutes at 4°C at $1200 \times g$. Approximately 300 mg of the pellet was re-suspended in 10 ml of Ham's F10 + 10% stripped calf serum (SCS, Gemini, Woodland, CA) containing 25 mg collagenase and incubated for 90 minutes at 37°C. The digestate was filtered through a 38- μ m stainless steel sieve and the supernatant spun as before. The pellet was dissolved in DMEM + 10% SCS. Cells were seeded and grown to confluence in Basal Medium (DMEM + 10%SCS + 1% ITS⁺; Becton-Dickinson/Colaborative Research, Bedford, MA).

Once confluent, the cultures were washed twice with Hanks' balanced salt solution to remove residual serum components and switched to serum-free defined medium (DM) consisting of DMEM + 1%ITS⁺ + 5 μ mol/L $FeSO_4$, 0.5 μ mol/L $ZnSO_4$, 1 nmol/L $CuSO_4$, 50 μ g/ml ascorbic acid, and 50 ng/ml epidermal growth factor. The experimental conditions were started by adding corresponding vehicle or steroid(s) with or without thrombin (0.5 IU) (American Diagnostica, Greenwich, CT) and/or factor VIIa (10 nmol/L) (Sigma). After a 48-hour incubation, conditioned DM was centrifuged, and the cells were washed with Hanks' balanced salt solution, lysed with 10 mmol/L Tris, (pH 7.4), 1 mmol/L sodium ortho-vanadate 1% SDS for protein analysis or Tri-Reagent (Sigma) for RNA analysis. Medium supernatants and cell lysates were stored at $-80^\circ C$ until used.

Human endometrial endothelial cells (HEECs) were isolated and cultured as previously described.¹² Briefly, HEECs were dissected free of visible vascular tissue, minced in Ca^{2+}/Mg^{2+} -free PBS (Cell Systems Corp., Kirkland, WA) + 1% dialyzed fetal bovine serum (FBS) (HyClone, Logan, UT), and digested with type VII high-purity collagenase (Sigma-Aldrich) and dispase (Boehringer Mannheim, Indianapolis, IN) in the presence of deoxyribonuclease (DNase) I (Sigma) and 1% dialyzed fetal bovine serum (FBS). The digest was filtered through a 70- μ m cell strainer (Falcon/Becton-Dickinson, Franklin Lanes, NJ) to remove undigested tissue fragments. The filtrate was treated with 1 mg/ml of biotinylated UEA-1 (*Ulex europaeus*) lectin (Sigma-Aldrich), which binds selectively to microvascular endothelial cells in human endometrial sections. Biotinylated UEA-1 labeled cells were separated from non-labeled cells by panning on activated surface/AIS MicroCELLector flasks (Applied Immune Sciences, Menlo Park, CA) coated with streptavidin (Sigma-Aldrich). Cells retained on the MicroCELLector flasks were released and cultured in phenol red-free DMEM:Ham's F-12 with 15 mmol/L HEPES, containing

endothelial cell growth factor supplemented with 15% stripped FCS in flasks coated with attachment factor (Cell Systems). The HEECs were grown to confluence, harvested by trypsin/EDTA and split 1:6 for four to six passages. The experimental conditions were carried out in a serum-free defined medium as described¹² following the addition of vehicle control, E_2 , MPA or thrombin (0.5 IU) for 48 hours. Conditioned DM was centrifuged, and the cells were washed with Hanks' balanced salt solution, lysed with 10 mmol/L Tris, (pH 7.4), 1 mmol/L sodium ortho-vanadate 1% SDS for protein analysis or Tri-Reagent (Sigma-Aldrich) for RNA analysis. Medium supernatants and cell lysates were stored at $-80^\circ C$ until used.

Human endometrial epithelial glandular cells (HEGEs) were derived from scraped endometrium as described for HESCs above. At the point where the stromal digestate is filtered, the sieve retains the glandular tissue. Approximately 25 mg of tissue is re-suspended in 15 ml BM and placed in a 10-cm petri dish for 1 hour in a 37°C incubator. The glands remain in the supernatant that is removed and distributed equally among polystyrene tissue culture dishes coated with 2% gelatin (Sigma).

ELISAs

These studies were performed at Regeneron Pharmaceuticals, Inc. (Tarrytown, NY). Ang-1 and Ang-2 protein concentrations in media derived from cultured HESCs or HEECs were measured by ELISA using recombinant Tie2 in the capture layer and an N-terminal antibody against Ang-1 or Ang-2 for reporting concentrations as previously described.¹³ All values were adjusted to the final protein concentration of the corresponding cell pellet determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA).

Microdissection

Human endometria were collected following hysterectomy for benign myomas from reproductive age women from the proliferative or early secretory phase. Endometria were collected only from women who had not received hormone treatment before hysterectomy. The endometrial samples were embedded in OCT compound (Tissue Tek, Sekura Finetek, Torrance, CA) for immediate snap-freezing in isopentane in liquid nitrogen and then stored at $-80^\circ C$ until the time of use. Frozen sections were cut with a cryostat (Reichert-Jung 2800 Frigocut, San Marcos, CA) set at $-20^\circ C$ to a thickness of 10 μ m and thaw-mounted onto superfrost slides (Fisher Scientific, Pittsburgh, PA). The microdissection system is based on ultrasound, using an Eppendorf-Microdissector (Westbury, NY) control device in combination with Narashige micromanipulators (Tokyo, Japan) and a Nikon microscope (Melville, NY). The microdissector uses ultrasound (25 to 60 kilohertz) to vibrate a glass probe as a cutting tool. An electronic pipette was used as an aspiration tool to recover the cut sample. These two functions are controlled by the Narashige three-dimen-

Figure 1. Representative cell microdissection. **A:** A 10- μ m frozen section from endometrium was stained as described in Materials and Methods. **B:** The glandular epithelium was separated out by microdissecting away the surrounding cells. **C:** The gland was subsequently removed with the aspiration tool.

sional coarse drive manipulators and micromanipulators. To dissect out the specific cell types of interest, frozen tissues were rapidly fixed in 4% paraformaldehyde (10 seconds) and stained for 5 seconds with hematoxylin QS (Vector Laboratories, Burlingame, CA) before microdissection. Briefly, the sectioned tissue was examined on an inverted Olympus BX51 microscope at $\times 200$ to 400 in Histosolve solution (Shandon, Inc., Pittsburgh, PA). The required cell type was identified by two investigators using a video monitor and the microdissector lowered onto the slide. The surrounding tissue was then isolated by dissection until the required cell group was secluded. Histosolve solution was then added to wash away debris and the aspiration pipette lowered adjacent to the isolate cell group while the vibrating microdissector was used to elevate the required cells from the slide. Once the cells were isolated the aspiration pipette was swiveled into position so that an Eppendorf tube could be placed over it and the cell contents expelled into the tube. Figure 1 shows sequential photos of an endometrial sample (A) from which a group of epithelial cells are isolated by microdissection (B) and are subsequently removed with the aspiration tool (C). Micro RNA isolation was immediately conducted on the extracted sample with the RNeasy kit (Qiagen, Valencia, CA).

Real Time-Quantitative RT-PCR

After assessing correct product formation by semi-quantitative RT-PCR, quantitative real time RT-PCR was conducted as follows: reverse transcription was carried out with AMV reverse transcriptase (Invitrogen, Carlsbad, CA). A quantitative standard curve was then created using a range of 500 pg to 250 ng of cDNA. The curve was created with the Roche Light Cycler (Roche, Indianapolis, IN) by monitoring the increasing fluorescence of PCR products during amplification. Once the standard curve was established, quantitation of the mRNA for the unknown samples was determined with the Roche Light Cycler and adjusted to the quantitative expression of β -actin from these same samples. Melting curve analysis was conducted to determine the specificity of the amplified products and the absence of primer-dimer formation.

All products obtained yielded the correct melting temperature. Figure 2 shows an example of the hybridization and the melting curve for β -actin derived from microdissected products. The following primers that have been previously reported^{14–17} were synthesized and gel-purified at the Yale DNA Synthesis Laboratory, Critical Technologies.

Figure 2. Hybridization and melting curve for β -actin. **Top:** The hybridization curve correlates the fluorescent output of Sybr Green with the PCR cycle number for several microdissected samples. PC, positive control; NC, negative control in which the RT step was omitted. **Bottom:** This melting curve analysis depicts the first negative derivative of the change in fluorescence over the change in temperature ($-dF/dT$) plotted versus melting temperature.

Figure 3. Ang-1 and Ang-2 expression by endometrial cells. The expression of Ang-1 and Ang-2 by freshly microdissected or cultured HEECs, HESCs, and HEGEs was assessed by real time-quantitative RT-PCR and differences among the group compared by Mann-Whitney test. **A:** Ang-1 expression by microdissected cells was greater among HESCs than the other cell types; $*P < 0.001$, $n = 3$. **B:** Ang-2 expression by microdissected cells was highest in HEECs compared to HESCs; $*P < 0.001$, $n = 3$; HEGEs: $**P < 0.05$, $n = 3$. **C:** Ang-1 expression by cultured HESCs was significantly higher than among HEECs and HEGEs; $*P < 0.001$, $n = 3$. **D:** Ang-2 expression by cultured HEECs was substantially higher than among HESCs and HEGEs; $*P < 0.001$, $n = 3$.

Statistics

The Sigma Stat 3.0 program (Jandell Scientific) was used to determine and conduct the appropriate statistical analysis. The tests used are included in the figure legends.

Results

Angiopoietin-1 and 2 Expression from Microdissected and Cultured Human Endometrial Cells

Because culture can result in changes in cell mRNA and protein expression, we initially conducted experiments in which individual cells representing the predominant endometrial populations were microdissected from frozen human endometria. As can be seen in Figure 3A, real time-quantitative RT-PCR demonstrated that Ang-1 mRNA is principally expressed by the HESCs and that the expression is statistically higher than the very low levels of Ang-1 expressed by the HEECs or HEGEs. By contrast, Figure 3B shows that minimal or no Ang-2 mRNA was detected in the HESCs and the HEGEs. However, significantly higher expression was detected in the HEECs. These *in vivo* results were compared to the relevant cell types in culture. The results in Figure 3, C and D show the same pattern of angiopoietin expression in cultured endometrial cells as was observed in microdissected cells. To assure that the correct cell type was isolated by microdissection, RT-PCR was conducted for ICAM, an endothelial specific endpoint. Figure 4 shows that only putative HEECs expressed ICAM whereas no ICAM transcript was observed for HEEGs or HESCs. Based on the findings in Figure 3, we focused our studies on the cells expressing the highest levels of Ang-1 or Ang-2, namely the HESCs and the HEECs.

Figure 4. ICAM-1 expression by microdissected endometrial cells. The expression of ICAM-1 by freshly microdissected HEECs, HEGEs, and HESCs from an early secretory endometrium was assessed by RT-PCR. The expression of β -actin by the same cells is also displayed. NC, negative control where the RT step was omitted.

Expression of Ang-1 by Cultured HESCs following Steroid Hormone Treatment

Previous studies from our laboratory have shown that HESCs express Ang-1 and that the expression of this protein is augmented by progestin.¹⁸ We have now quantitated the level of Ang-1 protein and mRNA expression in primary HESCs treated with E_2 or MPA. Figure 5 shows that both Ang-1 protein (left panel) and mRNA (right panel) expression is significantly induced following treatment with MPA compared to E_2 . No change in Ang-1 expression was noted between cultures treated with E_2 compared to vehicle control (not shown). Hence the level of protein expression as determined by ELISA is significantly enhanced by MPA (2.8 ± 0.67 ng/mg-protein) compared to E_2 alone (1.03 ± 0.28 ng/mg-protein). Analysis of mRNA by real time quantitative RT-PCR also demonstrated a significant induction of Ang-1 following MPA treatment (5.32 ± 1.28 Ang-1/ β -actin relative units) compared to E_2 alone (2.54 ± 0.47 Ang-1/ β -actin relative units).

Thrombin Reduces the Expression of Ang-1 by HESCs

We next studied the effects of thrombin and factor VIIa on the expression of Ang-1 by steroid hormone-treated

Figure 5. Quantitative effects of steroid hormones on the expression of Ang-1 by HESCs. The **left panel** depicts the effect of MPA (P) versus E_2 (E) treatment (Materials and Methods) on protein expression by HESCs whereas the **right panel** depicts the effects on Ang-1 mRNA. Progestin effects were statistically significant by Mann-Whitney test; $*P < 0.05$ for both protein and mRNA expression, $n = 8$.

Figure 6. Quantitative effects of thrombin and factor VIIa on Ang-1 expression by steroid hormone-treated HESCs. The **top panel** depicts the levels of Ang-1 protein expression and the **bottom panel** depicts the effects on Ang-1 mRNA following treatment with thrombin (Th), factor VIIa or both on E₂ (E) or MPA (P)-treated HESCs (Materials and Methods). Thrombin significantly inhibits Ang-1 protein and mRNA expression; Mann-Whitney test, **P* < 0.02, *n* = 9.

HESCs. As can be seen in Figure 6, thrombin significantly reduced the expression of Ang-1 protein (top panel) and mRNA (bottom panel) in HESCs treated with E₂ or with MPA. Thus E₂ + thrombin reduced the protein expression of Ang-1 to 0.29 ± 0.13 ng/mg-protein compared to 1.03 ± 0.28 ng/mg-protein for E₂ alone. Likewise, MPA + thrombin significantly reduced the levels of Ang-1 to 1.359 ± 0.609 ng/mg-protein compared to 2.811 ± 0.672 ng/mg-protein with MPA alone. Similarly at the mRNA transcript level, E₂ + thrombin significantly reduced the expression of Ang-1 mRNA to 0.66 ± 0.23 relative units from 2.544 ± 0.465 in cultures treated with E₂ alone. Likewise, MPA + thrombin significantly reduced the levels of Ang-1 mRNA to 1.776 ± 0.351 relative units from 5.319 ± 1.284 in cultures treated with MPA alone. The quantitative RT-PCR values were adjusted for the expression of β -actin.

As can also be seen in Figure 6, neither HESC Ang-1 protein nor mRNA levels were affected by exposure to VIIa. Furthermore, the addition of VIIa to thrombin did not result in any further changes above those observed following treatment by thrombin alone. To confirm the specificity of thrombin effects and rule out the possibility of proteolytic activity, the expression of plasminogen activator inhibitor-1 (PAI-1) following treatment of HESCs with thrombin was assessed in parallel. As previously reported,¹⁹ levels of PAI-1 mRNA expression were increased by thrombin in these samples (data not shown).

Figure 7. Quantitative effects of thrombin on Ang-2 expression by control and steroid hormone-treated HEECs. The **top panel** depicts the levels of Ang-2 protein expression and the **bottom panel** depicts the effects on Ang-2 mRNA following HEEC treatment with thrombin. Vehicle control (C), E₂ (E), or MPA (P). Thrombin exerted statistically significant effects only on Ang-2 protein expression; one-way analysis of variance; **P* < 0.05, *n* = 5.

Expression of Ang-2 by HESCs

Consistent with our previous results,^{18,20} levels of HESC Ang-2 protein and mRNA were low compared to expression by HEECs (Figure 3). Neither thrombin nor VIIa affected Ang-2 expression in these cells (data not shown).

Expression of Ang-1 by HEECs

As previously reported,¹⁸ very low levels of Ang-1 protein and mRNA were expressed by cultured HEECs (Figure 3). Since controversy exists as to the presence of estrogen and progesterone receptors in these cells, HEECs were treated with control, E₂, or MPA and the production of Ang-1 analyzed. Virtually no Ang-1 mRNA or protein expression was noted in response to steroids (data not shown).

Expression of Ang-2 by HEECs

HEECs have previously been shown to express Ang-2 protein and mRNA.^{18,20} Thrombin treatment resulted in a small decrease in Ang-2 expression in control-treated HEECs that was significant at the protein level (thrombin = 13.70 ± 0.97 ng/mg-protein *versus* control = 19.06 ± 1.31 ng/mg-protein) but not at the mRNA transcript levels (thrombin = 87.50 ± 15.88 relative units *versus* control = 94.44 ± 14.67 relative units) (Figure 7). Once again, treatment with E₂ or MPA had no significant effect on the expression of Ang-2 compared to controls.

Discussion

Rogers and Abberton²¹ have noted conflicting results among published studies on the cellular localization of endometrial expression of Ang-1 and Ang-2. To resolve this controversy, we carried out microdissection of endometrial stromal, glandular, or endothelial cells from snap-frozen endometria derived from hysterectomy specimens. While the endometria are not from "normal" women, we chose the most benign condition for which hysterectomies are performed, namely non-hemorrhagic myomatous uteri, to reflect the most physiological endometria one could ethically obtain from humans. We then conducted RNA isolation followed by quantitative real time RT-PCR. These microdissection results were consistent with the *in vitro* observations that the predominant expression of Ang-1 occurs in HESCs, while the predominant expression of Ang-2 occurs in HEECs. While others have reported that a significant expression of Ang-1 and/or Ang-2 can occur by circulating and/or resident endometrial leukocytes in the late secretory phase of the cycle,²² we attempted to avoid leukocytes by only performing microdissection of proliferative or early secretory endometria. In addition, previous studies showed that cultured HESCs did not display expression of CD45, a specific leukocyte marker.²³

Interestingly, the expression of Ang-1 mRNA was higher from microdissected compared to cultured cells. We attribute this to the possibility that culture *per se* may lead to decreased levels of Ang-1 expression as these cells are not in contact with other cell types normally found in whole tissues, eliminating potential paracrine interactions. Most importantly, the profile of Ang-1 expression remained the same *in vivo* and *in vitro*, with the highest expression of Ang-1 by the HESCs and the highest expression of Ang-2 by the HEECs.

Having established this observation, the present study sought to characterize the effects of thrombin on the expression of Ang-1 and Ang-2 by these cells. Thrombin exerts both proteolytic and cellular actions that include hemostasis and angiogenesis, respectively.^{24,25} Angiogenesis plays a key role in a broad array of physiological and pathological processes. Although the formation of thrombin is necessary for hemostasis, aberrant expression of this protein results in thrombosis. For example, both acquired and inherited thrombophilias are associated with maternal venous thrombotic events as well as fetal death and abruptio.^{26–28}

In addition to its role in clotting, we have previously showed that thrombin enhances expression of the potent angiogenic factor, VEGF, in cultured HESCs.⁶ In contrast, this study shows that thrombin significantly reduced the expression of Ang-1 protein and mRNA expression by MPA-treated HESCs reflecting a hormonal milieu that would be observed in women treated with long-term progestin-only contraception such as Depo-Provera (medroxyprogesterone acetate, Pfizer, Cambridge, MA). Since Ang-1 is a key factor in vessel stabilization, inhibition of its expression by thrombin could be expected to enhance pathological angiogenesis. Indeed, women undergoing long-term progestin-only contraception display

a high incidence of abnormal bleeding from enlarged fragile vessels and such bleeding is likely to result in thrombin production. Thus, our findings may account for the continued bleeding observed under these conditions as the resultant thrombin formation would inhibit Ang-1 production by the stroma that surround the endometrial vessels.

Prior studies suggest that use of long-term progestin-only contraception results in focal areas of hypoxia,^{18,29} and this in turn, decreases the expression of Ang-1.²⁹ Thus, two events, namely hypoxia and thrombin formation following abnormal endometrial bleeding, may account for a continued cycle that furthers pathological angiogenesis.

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