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Expression of Angiopoietin-2 by Human Endometrial Endothelial Cells: Regulation by Hypoxia and Inflammation

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The functional endometrial layer receives the implanting blastocyst, but is sloughed off during menstruation. Angiogenesis regulates growth and repair of cycling human endometrium. While vascular endothelial growth factor initiates angiogenesis, the angiopoietins (Angs) acting via the Tie2 receptor, are key regulators of subsequent angiogenic steps. This study is the first to localize Ang-2 and Tie2 in human endometrium and to study Ang-2 regulation in cultured human endometrial endothelial cells (HEECs). Immunohistochemistry revealed that expression of Ang-2 and Tie2 was absent from the glands, low in stromal cells, and intense in the endothelial cells. In contrast, only weak expression of Ang-1 was detected. The phase of the menstrual cycle did not appear to affect the expression of Ang-2 or Tie2. *In vitro* studies were carried out utilizing isolated HEECs, the most relevant model for endometrial microvascular biology studies. Both hypoxia and phorbol-myristate-acetate enhanced Ang-2 mRNA levels in HEECs. These results suggest that Ang-2 plays a role in endometrial pathologies complicated by impaired blood flow and inflammation.

Angiogenesis in the adult is commonly observed in wound healing and in cases of diabetic retinopathy, rheumatoid arthritis or tumor growth (1, 2). In contrast, physiologic angiogenesis is observed in the female reproductive tract where it plays key roles in the cycling ovary and endometrium (for reviews see 3 and 4). At times however, endometrial pathologies result in abnormal uterine bleeding. Indeed, abnormal uterine bleeding is a leading indication for emergency office visits and hysterectomies (3). Moreover, it is the primary factor resulting in discontinuation of long-term, progestin-only contraceptives (5, 6). Vascular endothelial growth factor (VEGF) and its receptors have been postulated to be key regulators of vascular regeneration in the human endometrium under normal and pathological conditions (3, 4). However, a second family of proteins, exerting a later but equally important role in vascular development and stabilization, have now been identified and termed the angiopoietins (Angs) (7). Angs regulate angiogenesis by activating or by blocking the activation of Tie2, a surface receptor tyrosine kinase generally restricted to endothelial cells (7–9). Two members of this family, Ang-1 and Ang-2 share 60% homology (10, 11). Ang-1 affects vascular integrity and is involved in vessel maturation. Transgenic Ang-1 or Tie2 knockout mice result in embryonic lethality associated with aberrant interactions between endothelial cells and their surrounding supporting cells (12). Moreover, transgenic over-expression of Ang-1 elicits marked pathological hypervascularization and regional varicosities (13). Consistent with the action of Ang-2 as a partial antagonist of Ang-1/Tie2 interactions, transgenic over-expression of Ang-2 results in embryonic lethality with a similar phenotype as mice lacking either Ang-1 or Tie2 (11). Ang-2 is expressed in areas undergoing vascular remodeling and is involved in neovascularization (11). Like VEGF, Ang-2 enhances vascular permeability. By competing with Ang-1 for binding to their common receptor Tie-2, Ang-2 ultimately results in decreased vessel maturation and either vessel regression (in the absence of VEGF) or enhanced vessel sprouting (in the presence of VEGF). Both hypoxia and VEGF up-regulate Ang-2 expression in bovine microvascular endothelial cells (14). In short, VEGF, Ang-1 and Ang-2 have complementary roles in vascular development and maintenance. The expression and regulation of Ang-1 and Ang-2 may be critical in the balance between physiologic and pathologic angiogenesis. Thus, we evaluated immunohistochemical staining for Ang-1, Ang-2 and Tie2 in the endometrium and evaluated the effects of

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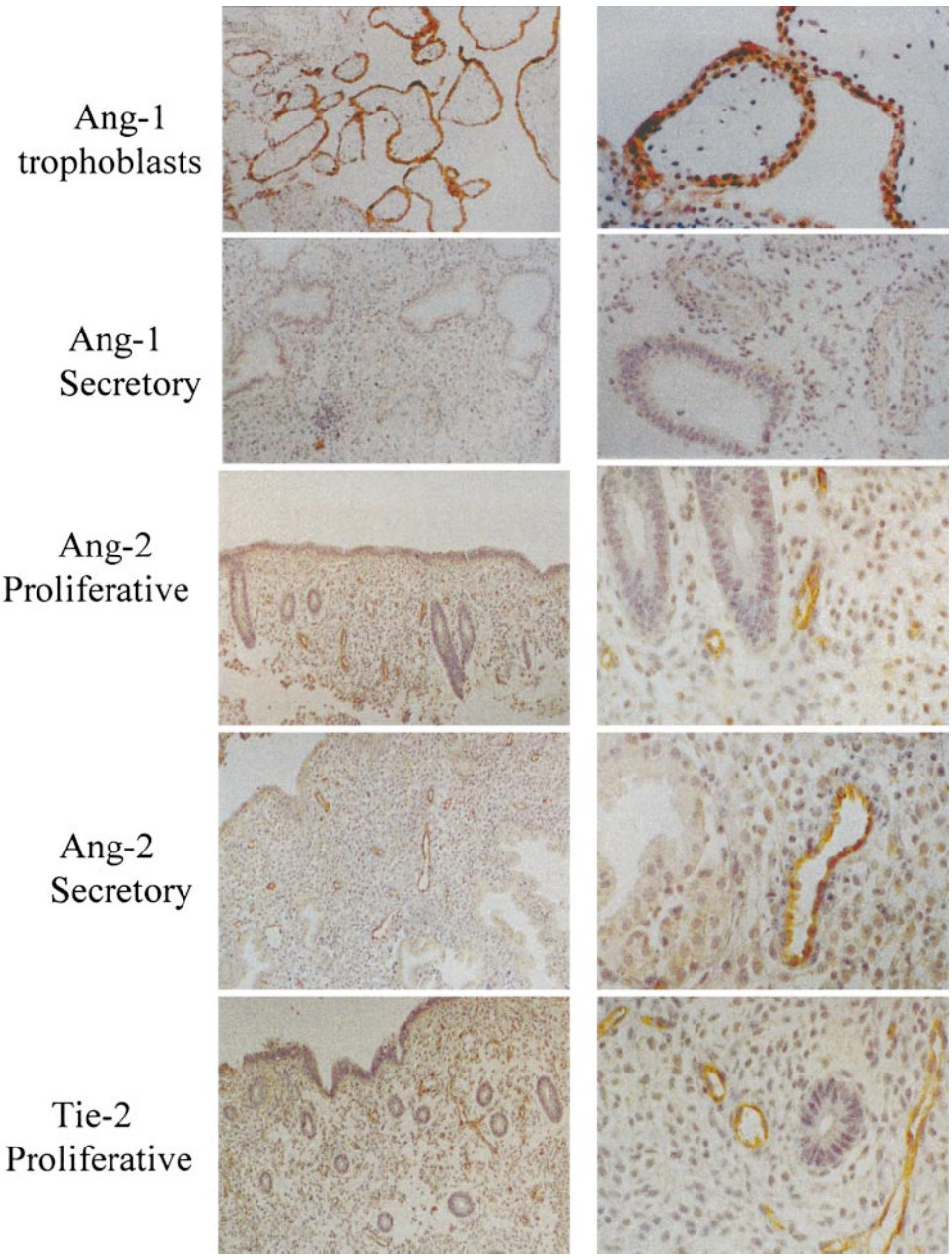

FIG. 1. Immunohistochemistry of Ang-1 Ang-2 and Tie 2 in human endometrium. Immunohistochemical staining for Ang-1, Ang-2 and Tie 2 in proliferative and/or secretory phase human endometria were carried out in paraffin fixed sections as described under Materials and Methods. First trimester trophoblasts were also stained as a positive control for Ang-1. Magnification on the left panel is ×100 and on the right panel is ×400. Antigens are identified by brown peroxidase staining.

hypoxia and an inflammatory mediator in cultured human endometrial endothelial cells (HEECs).

MATERIALS AND METHODS

Cell culture. Endometrial endothelial cells were grown to confluence on flasks coated with attachment factor (Cell Systems, Kirkland, WA) in CS-C Complete Medium supplemented with 15% stripped fetal calf serum as described (15). The cells were harvested by trypsin/EDTA and split 1:6 for passaging. Experimental incubations involved starving confluent cells for 3 h in M 199 (Gibco, Grand Island, NY) in a standard incubator. The medium was exchanged for a serum free defined medium lacking growth factors. In some experiments HEECs were treated with PMA, 10^{-8} M estradiol (E_2), 10^{-7} M medroxyprogesterone acetate (MPA) or E_2 + MPA for 4 h prior to harvesting. For studies involving hypoxia, cultured HEECs were placed in sealed chambers containing a portable gas oxygen analyzer and a beaker of water to maintain humidity. The chambers were purged with 5% CO_2, 95% N_2, and purging continued for an additional 15 min after the oxygen analyzers read 0–1% O_2 (12–14 mm Hg). The sealed chambers were placed in a standard 37°C incubator

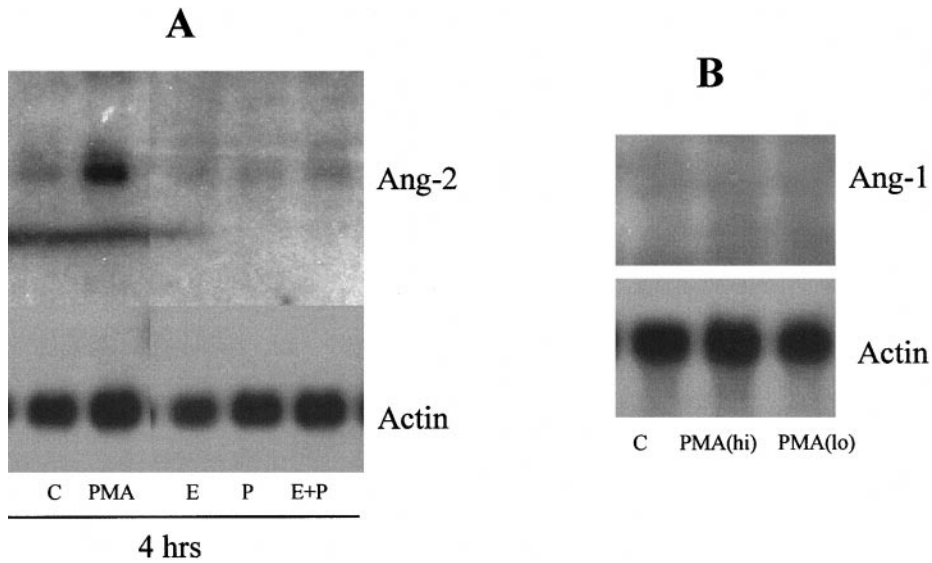

FIG. 2. Effect of PMA on Ang-1 and Ang-2 mRNA expression. Cultured HEECs were treated for 4 h with (**A**) control media (C), or media containing 4 ng/ml PMA, 10^{-8} M E_2 (E), 10^{-7} M MPA (P) or E_2 + MPA (E + P) (**B**) control media (C), 25 ng/ml PMA (hi) and 4 ng/ml PMA (lo) as described under Materials and Methods. Northern blots were performed for Ang-1, Ang-2 and loading efficiencies assessed with Actin.

for 48 h. Experimental incubations were terminated by removing the conditioned media and extracting the RNA as described (16) with RNA Isolator (Genosys, The Woodlands, TX).

Immunohistochemistry. This was performed in paraffin fixed sections of proliferative or secretory phase human endometria. Peroxidase staining was conducted with the ABC elite kit from Vector Laboratories (Burlingame, CA) as described (16). Goat polyclonal antibodies to Ang-2 and rabbit polyclonal antibodies to Tie-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Rabbit polyclonal antibody to Ang-1 was obtained from Alpha Diagnostic (San Antonio, TX).

Northern analysis. RNA was separated on 1% agarose gels and transferred to Zeta-Probe Nylon membranes (Bio-Rad, Hercules, CA). The probe for Ang-1 was a 1.4 kb RT-PCR fragment containing the open reading frame as described by Davis *et al.* (17) and the probe for Ang- 2 was generated by RT-PCR of total human placental RNA utilizing MuLV reverse transcriptase (Perkin Elmer, Gaithersburg, MD), and oligo $d(T)_{16}$. The primers for the cDNA probe for Ang-2 (bases 1346–1798) were selected from the published mRNA sequences (GenBank) as follows: forward, 5′- GTTGATTTTCAGAGGACTTGG-3′ and reverse, 5′-CGAATAGCCTGAGCCTTTCCA-3′.

PCR was conducted with AmpliTaq DNA polymerase (Perkin Elmer) as follows: 1.75 min at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The reaction was then continued at 72°C for 7 min. The product was analyzed on agarose gel and the corresponding band (453 bp) extracted. Probes were labeled with ^{32}P-dCTP, to high specific activity by random priming with a Boehringer-Mannheim kit (Nutley, NJ). Total RNA loads were standardized by reprobing the stripped membranes with actin as previously described (18).

RESULTS AND DISCUSSION

As can be seen in Fig. 1, immunostaining for Ang-2 was intense in the endothelial cells, almost absent from stromal cells and completely absent in epithelial cells. Ang-2 was expressed at similar levels for the proliferative and secretory phases of the menstrual cycle. Figure 1 also demonstrates that the expression of Tie-2 was very similar to that observed with Ang-2 with predominant staining in the endothelial cells. Conversely, Ang-1 was weakly present in endometrial stromal and endothelial cells (Fig. 1). To assess the specificity of the Ang-1 antibody, first trimester placenta was stained as a control. As recently demonstrated (19) intense Ang-1 staining was detected in the cyto/syncytiotrophoblast bilayer (Fig. 1). These results are consistent with previous findings demonstrating that in the developing mouse embryo, Ang-1 was mainly expressed in cells adjacent to endothelial cells (10, 11, 20), while Ang-2 was expressed in the periendothelial as well as endothelial cells (11). Moreover, a recent study has demonstrated expression of Tie-2 and Ang-2 mRNA in villous endothelial cells of human placenta (21). Additionally, Tie 2 mRNA was detected in third trimester decidual endothelial cells (21).

Figure 2 demonstrates the expression of Ang mRNA by cultured HEECs. Consistent with the lack of effect by steroids observed *in vivo* (Fig. 1), neither estradiol nor the synthetic progestin, medroxyprogesterone acetate (MPA), enhanced Ang-2 expression in cultured HEECs (Fig. 2A). In contrast, the inflammatory agent PMA induced HEEC Ang-2 mRNA expression 20-fold compared to control treatment, suggesting that inflammatory mediators can directly enhance endometrial angiogenesis. Contrary to that observed for Ang-2, the levels of Ang-1 mRNA were barely above background intensity regardless of the length of exposure times. Furthermore, neither low nor high doses of PMA were capable of inducing the expression of Ang-1 mRNA (Fig. 2B).

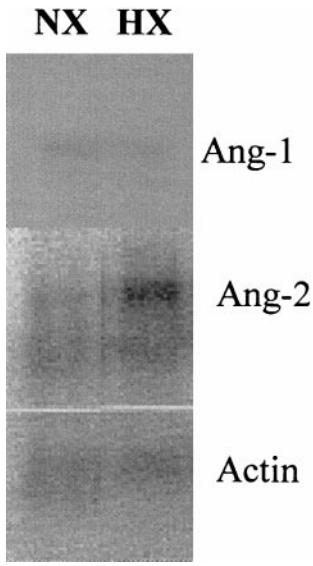

FIG. 3. Effect of hypoxia on Ang-1 and Ang-2 mRNA expression. Cultured HEECs were treated under normoxia (**NX**) or hypoxia (**HX**) as described under Materials and Methods. Northern blots were performed for Ang-1 and Ang-2 and loading efficiencies assessed with Actin.

Since endometrial bleeding often results in local hypoxia (22), experiments were conducted in which isolated HEECs were exposed to normoxia or hypoxia for 48 h. Figure 3 demonstrates that mRNA levels of Ang-2 in HEECs exposed to hypoxia were induced by 15 fold. In accordance with these findings, previous reports have shown that tumor necrosis factor-α upregulated Ang-2 expression in human umbilical vein endothelial cells (23) and that hypoxia induced Ang-2 production by bovine microvascular endothelial cells (14, 24).

In contrast to our findings with Ang-2, the expression of Ang-1 mRNA expression by HEECs either under normoxia or hypoxia was low. The mRNA results observed in Figs. 2 and 3 are consistent with the expression patterns for the Angs observed with immunohistochemical staining.

In the presence of VEGF, the primary role of Ang-2 involves vessel sprouting and remodeling (8). In the absence of VEGF, however, Ang-2 expression results in vessel regression and atrophy (8). Thus, Ang-2 is a key mediator in the balance between negative and positive angiogenic regulation. We postulate that "fragile" vessels arise from aberrant angiogenesis and vessel maintenance and that patients displaying abnormal uterine bleeding resulting from inflammation, anovulation, neoplasia and long term progestin only contraceptives will display induced expression of Ang-2 in the endometrium.

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REFERENCES

1. Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1,** 27–31.
2. Ferrara, N. (1999) Molecular and biological properties of vascular endothelial growth factor. *J. Mol. Med.* **77,** 527–543.
3. Smith, S. K. (1998) Angiogenesis, vascular endothelial growth factor and the endometrium. *Hum. Reprod. Update* **4,** 509–519.
4. Torry, D. S., and Torry, R. J. (1997) Angiogenesis and the expression of vascular endothelial growth factor in endometrium and placenta. *Am. J. Reprod. Immunol.* **7,** 21–29.
5. Belsey, E. M. (1988) The association between vaginal bleeding patterns and reasons for discontinuation of contraceptive use. *Contraception* **38,** 207–225.
6. Su-Juan, G., Ming-Kun, D., Ling-De, Z., Ying-Lin, L., Shu-Hua, W., and Sivin, I. (1994) A 5-year evaluation of NORPLANT contraceptive implants in China. *Obstet. Gynecol.* **83,** 673–678.
7. Gale, N. W., and Yancopoulos, G. D. (1999) Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev.* **13,** 1055–1066.
8. Holash, J., Wiegand, S. J., and Yancopoulos, G. D. (1999) New model of tumor angiogenesis: Dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* **18,** 5356–5362.
9. Valenzuela, D. M., Griffiths, J. A., Rojas, J., Aldrich, T. H., Jones, P. F., Zhou, H., McClain, J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Huang, T., Papadopoulos, N., Maisonpierre, P. C., Davis, S., and Yancopoulos, G. D. (1999) Angiopoietins 3 and 4: Diverging gene counterparts in mice and humans. *PNAS* **96,** 1904–1909.
10. Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonpierre, P. C., and Yancopoulos, G. D. (1996) Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **87,** 1161–1169.
11. Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N., Daly, T. J., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277,** 55–60.
12. Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87,** 1171–1180.
13. Suri, C., McClain, J., Thurston, G., McDonald, D. M., Zhou, H., Oldmixon, E. H., Sato, T. N., and Yancopoulos, G. D. (1998) Increased vascularization in mice overexpressing angiopoietin-1. *Science* **282,** 468–471.
14. Oh, H., Takagi, H., Suzuma, K., Otani, A., Matsumura, M., and Honda, Y. (1999) Hypoxia and vascular endothelial growth factor selectivelyup-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J. Biol. Chem.* **274,** 15732–15739.
15. Schatz, F., Soderland, C., Hendricks-Munoz, K. D., Gerrets, R. P., and Lockwood, C. J. (2000) Human endometrial endothelial cells: Isolation, characterization, and inflammatory-mediated expression of tissue factor and type 1 plasminogen activator inhibitor. *Biol. Reprod.* **62,** 691–697.
16. Lockwood, C. J., Nemerson, Y., Guller, S., Krikun, G., Alvarez, M., Hausknecht, V., Gurpide, E., and Schatz, F. (1993) Progestational regulation of human endometrial stromal cell tissue factor expression during decidualization. *J. Clin. Endo. Metab.* **76,** 231–236.
17. Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maison-

pierre, P. C., and Yancopoulos, G. D. (1996) Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **87,** 1161–1169.

18. Seligman, S. P., Nishiwaki, T., Kadner, S. S., Dancis, J., and Finlay, T. H. (1997) Hypoxia stimulates ecNOS mRNA expression by differentiated human trophoblasts. *Ann. NY Acad. Sci.* **828,** 180–187.
19. Dunk, C., Shams, M., Nijjar, S., Rhaman, M., Qiu, Y., Bussolati, B., and Ahmed, A. (2000) Angiopoietin-1 and angiopoietin-2 activate trophoblast tie-2 to promote growth and migration during placental development *Am. J. Pathol.* **156,** 2185–2199.
20. Kim, I., Kim, J. H., Ryu, Y. S., Jung, S. H., Nah, J. J., and Koh, G. Y. (2000) Characterization and expression of a novel alternatively spliced human angiopoietin-2. *J. Biol. Chem.* **275,** 18550–18556.
21. Goldman-Wohl, D. S., Ariel, I., Greenfield, C., Lavy, Y., and Yagel, S. (2000) Tie-2 and angiopoietin-2 expression at the fetal-maternal interface: A receptor ligand model for vascular remodelling. *Mol. Hum. Reprod.* **6,** 81–87.
22. Sharkey, A. M., Day, K., McPherson, A., Malik, S., Licence, D., Smith, S. K., and Charnock-Jones, D. S. (2000) Vascular endothelial growth factor expression in human endometrium is regulated by hypoxia. *J. Clin. Endocrinol. Metab.* **85,** 402–409.
23. Kim, I., Kim, J. H., Ryu, Y. S., Liu, M., and Koh, G. Y. (2000) Tumor necrosis factor-alpha upregulates angiopoietin-2 in human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.* **269,** 361–365.
24. Mandriota, S., and Pepper, M. S. (1998) Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ. Res.* **83,** 852–859.