Estrogen-dependent Production of Erythropoietin in Uterus and Its Implication in Uterine Angiogenesis*

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Although erythropoietin (Epo) has been shown to possess in vitro angiogenic activity, its physiological significance has not been demonstrated. Normally angiogenesis does not occur actively in adults but an exception is the female reproductive organ. In the uterine endometrium, angiogenesis takes place actively for supporting the endometrial growth that occurs during transition from the diestrus to estrous stage. This transition is under control of 17β-estradiol (E2), an ovarian hormone, and can be mimicked by injection of E2 to ovariectomized (OVX) mouse. Thus, the uterus is a pertinent site to examine the Epo function in angiogenesis. We found that Epo protein and its mRNA were produced in an E2-dependent manner, when the uterus from OVX mouse was cultured in vitro. The de novo protein synthesis was not needed for E2 induction of Epo mRNA. Administration of E2 to OVX mouse induced a rapid and transient increase in Epo mRNA in the uterus. Injection of Epo into the OVX mouse uterine cavity promoted blood vessel formation in the endometrium. Furthermore, injection of the soluble Epo receptor capable of binding with Epo into the uterine cavity of non-OVX mouse in diestrus stage inhibited the endometrial transition to proestrus stage, whereas heat-inactivated soluble Epo receptor allowed the transition to occur. These results, combined with our finding that the endothelial cells in uterine endometrium express Epo receptor, strongly suggest that Epo is an important factor for the E2-dependent cyclical angiogenesis in uterus.

Angiogenesis is the formation of new blood vessels by the extension of pre-existing vessels into avascular area and involves the proteolytic degradation of the vascular basal membrane, proliferation and migration of endothelial cells, and alignment of the migrating cells for tubular formation. Angiogenesis occurs very actively in embryogenesis, but it is down-regulated in the healthy adult. Active neovascularization in adults takes place in certain pathological conditions such as arthritis, diabetic retinopathy, wound healing, and tumor growth (reviewed in Ref. 1). An exception in adults is the female reproductive organ, where active angiogenesis is demanded to support the cyclic remodeling of tissues. In every estrus cycle, capillary networks in the ovaries are formed for supporting development of follicles and corpora lutea. In the uterus, cyclic formation of blood vessels in the functional endometrium occurs to compensate for the lost vessels. In response to embryonic implantation, decidual transformation of the endometrium is accompanied by neovascularization, which ultimately leads to formation of maternal vessels in the placenta.

Cyclic development of the uterine endometrium is under the control of E2, which is produced by ovarian follicles (2). This endometrial development can be mimicked by the administration of E2 to the OVX immature or adult animals (3–5). A number of growth factors including fibroblast growth factor, tumor growth factor, and VEGF have been implicated in angiogenesis (Refs. 6–8 and references therein). One of the early events caused by the E2 administration to the OVX rats is the increased vascular permeability in the endometrium (9). Based on the temporal pattern of mRNA expression after the E2 administration (10) and capability of increasing vascular permeability as well as the mitogenic activity for vascular endothelial cells (11–13), VEGF has been proposed to be a critical factor in the early phase of E2-induced angiogenesis (10, 14).

Epo is a key factor for regulating erythropoiesis by stimulating proliferation and differentiation of late erythroid precursor cells (15–19). Epo involved in erythropoiesis is produced by the kidney in adults and the liver in fetuses. In addition to the erythropoietic function of Epo, we and others (20–26) have recently shown that the brain has a paracrine Epo/EpoR system, which is independent of erythropoietic system; neurons express EpoR (20, 22) and astrocytes produce Epo (24–26). We have shown (22, 27) that brain Epo contributes to neuron survival by protecting neurons from ischemic damage.

Angiogenic activity of Epo has been studied by the use of in vitro cultured endothelial cells. EpoR mRNA is expressed in endothelial cells from human umbilical vein, bovine adrenal capillary, and rat brain capillary (28, 29). Epo stimulates proliferation and migration of human and bovine endothelial cells (30) and also angiogenesis of the rat thoracic aorta (31). Recent studies of human umbilical vein endothelial cells indicate that Epo signaling in endothelial cells is conducted via tyrosine phosphorylation of proteins including phosphorylation of transcription factor STAT-5, which is similar to that in erythroid

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1 The abbreviations used are: E2, 17β-estradiol; OVX, ovariectomized; VEGF, vascular endothelial growth factor; Epo, erythropoietin; EpoR, erythropoietin receptor; sEpoR, soluble EpoR; RT-PCR, reverse transcription-polymerase chain reaction.
cells (32). However, it is unknown whether endothelial EpoR is physiologically functional or is only a vestige reflecting a common developmental lineage between endothelial cells and hematopoietic cells (8). The uterus where the active angiogenesis takes place in an E2-dependent manner may be a target pertinent to examine the physiological significance of Epo in angiogenesis. We found E2-dependent Epo production in uterus, suggesting the estrous cycle-dependent fluctuation of Epo concentration in the uterine tissues. This finding prompted us to examine the role of Epo in the E2-induced endometrial regeneration with an expectation that Epo acts as a uterine angiogenic factor.

EXPERIMENTAL PROCEDURES

Epo, Epo Assay, and sEpoR—Recombinant human Epo was produced and isolated as described previously (33, 34). Epo was measured with a sandwich-type enzyme-linked immunoassay using two monoclonal antibodies that bind Epo at different epitopes (35). This assay measures Epo as low as 1 pg/ml. Recombinant human Epo was used as a standard. Recombinant murine soluble EpoR (sEpoR), an extracellular domain of EpoR capable of binding with Epo, was produced and isolated as described in the previous report (36).

RT-PCR—Total RNA was prepared by the use of RNA Isolation System kit (Promega). The RT reaction was performed using a random nonamer primer and 1 μg of RNA in a volume of 20 μl. PCR primers of Epo and β-actin were those described previously (22, 26), and those of VEGF were sense primer (mVEGF130F, 5’-TGCTGTACCTCCACCATGCCAA-3’) and antisense primer (mVEGF657R, 5’-ACCGCCTTGGCTTGTCACATCT-3’) (GenBank™ accession number M95200; Ref. 48). PCR cycles and conditions for denaturation, annealing, and elongation were 40 cycles, 1 min at 94 °C, 2 min at 63 °C, and 3 min at 72 °C of Epo; 25 cycles, 1 min at 94 °C, 2 min at 64 °C, and 3 min at 72 °C of VEGF; 25 cycles, 1 min at 94 °C, 2 min at 61 °C, and 3 min at 72 °C of β-actin. The amplified DNA was fractionated by electrophoresis and stained with ethidium bromide. Band intensity was measured for only the most abundantly expressed VEGF mRNA. Bars represent mean ± S.D. (n = 5).

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FIG. 1. *In vitro* cultured uterus from OVX mouse produces Epo protein and its mRNA in an E2-dependent manner. As described under “Experimental Procedures,” one horn of the uterus from an OVX mouse was cultured in the medium containing E2 for 6 h, and the contralateral horn was cultured without E2 as a control. Epo secreted in the culture media was measured, and the tissues were used for detection of Epo mRNA by RT-PCR. a, E2-dependent production of Epo protein; b, effects of 200 μM cycloheximide (CHX) and 10 μM actinomycin D (Act D) on 10−7 M E2-dependent expression of Epo mRNA and Epo protein. Each value indicates the average Epo of duplicate cultures.

FIG. 2. E2 induces *in vivo* expression of Epo and VEGF mRNAs in the uterus of OVX mice. At intervals after injection of E2 to OVX mice, uteri were removed for semi-quantitative measurement of Epo, VEGF, and β-actin mRNAs by RT-PCR (see “Experimental Procedures”). a, Epo mRNA-derived product; b, VEGF-derived product. The amplified bands of two individual mice are shown. Lanes C (open columns) indicate controls; mice given vehicle. Closed columns indicate band intensities relative to the intensity (open column) of controls. Three VEGF products are derived from alternative splicing (39, 40). Band intensity was measured for only the most abundantly expressed VEGF mRNA. Bars represent mean ± S.D. (n = 5).
Culture of the Uterus from OVX Mouse—Bilateral horns of the uterus from OVX mouse were cut into two separate horns. One horn was cultured in the medium containing E2 for 6 h in a humid 5% CO2 atmosphere at 37 °C in phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 20% charcoal-treated fetal calf serum, and the contralateral horn was cultured without E2 as a control. Epo protein secreted in the culture media was measured. Tissues were used for detection of Epo mRNA by RT-PCR.

Administration of E2 and Epo to OVX Mouse—E2 (0.5 mg/kg) or vehicle (olive oil) was given intraperitoneally. Epo was injected into the uterine cavity of the OVX mouse as follows. The abdominal wall of the mouse under deep anesthesia was incised, and the entire uterus was pulled out. Then the uterus was ligated at the oviduct and vaginal ends to form three uterine cavities, two lateral and one central. Epo in 100 μl of saline was injected into one of the lateral cavities through a microsyringe with a 32-gauge needle (Hamilton) and saline was injected into the contralateral cavity as a control. At 24 h after injection, the uterus was excised and fixed in Zamboni solution.

Immunohistochemistry—Methods for preparing cryosections and staining tissues were as described previously (37). Vascular endothelial cells and EpoR-expressing cells were detected by the use of anti-factor VIII (von Willebrand factor) antiserum (Dako, 1:200) and anti-N-terminal EpoR antiserum (38), respectively. Immunocomplexes were visualized using ABC kit (Vector) and diaminobenzidine (Dojin).

RESULTS

E2-dependent Production of Epo and Its mRNA by the in Vitro Cultured Uterus—If we assume that Epo plays an important role in E2-dependent angiogenesis in the uterine endometrium, there would be two possibilities for Epo production. One possibility is that uterine target cells gain responsiveness to Epo by the action of E2, and the serum Epo derived from the kidney acts on the E2-sensitive cells. The other would be that a local site for Epo production exists in the uterine tissues, and the production is induced by E2, resulting in an E2-dependent increase of Epo concentration in uterine tissues sufficient for activating angiogenesis. To test the latter possibility, we first examined if the in vitro cultured uterus from OVX mouse produced Epo protein and its mRNA in an E2-dependent manner. One of the bilateral horns of the uterus prepared from the OVX mouse was cultured in the medium containing E2, and the other horn was cultured in the absence of E2 as a control. Epo production into the culture medium was almost undetectable in...
the absence of E₂, whereas the culture with E₂ produced Epo in an E₂-dependent manner, and the increase was evident at physiological concentrations of E₂ (10⁻⁸ M) (Fig. 1c). Cycloheximide and actinomycin D completely inhibited Epo-induced production of Epo by the cultured uterus (Fig. 1b), demonstrating that Epo secreted into the culture media was newly synthesized. Epo mRNA was detected with RT-PCR, and restriction mapping confirmed that the amplified DNA band was the specific product derived from Epo mRNA (data not shown). Epo mRNA was definitively expressed in the uterus cultured with E₂, but it was undetectable in that cultured without E₂ (Fig. 1b). Actinomycin D completely blocked expression of Epo mRNA, whereas the expression was superinduced by cycloheximide, indicating that the de novo protein synthesis is not needed for induction of Epo mRNA. Epo that supports erythropoiesis is produced by the kidney in a hypoxia-inducible manner (17, 18). We cultured the uterus in the absence or presence of 10⁻⁷ M E₂ in 21, 5, and 2% O₂, but Epo production was not activated by the low oxygen concentrations (data not shown).

**In Vivo E₂-induced Expression of Epo mRNA in the Uterus**—We examined whether or not E₂ injection to the OVX mouse induced Epo mRNA in the uterus. E₂ was given intraperitoneally, and the uterus was removed at intervals to extract RNA. Epo and β-actin mRNAs were measured semiquantitatively by setting PCR cycles to be approximately proportional to the band intensity of the amplified DNA. The two upper panels in Fig. 2a show Epo mRNA- and β-actin mRNA-derived products from two individual mice, and the lowest panel in Fig. 2a indicates band intensities of Epo mRNA-derived product (n = 5 mice). There was a clear increase of Epo mRNA at 1 h after E₂ injection, and the increase continued for at least 4 h, but at 8 h its level decreased to that of E₂-uninjected mice. VEGF mRNA was also increased at 1 h after E₂ injection, and thereafter its level was gradually reduced (the lowest panel in Fig. 2b), which was in agreement with the previous finding (10). Three amplified DNA bands (the most upper panel in Fig. 2b) are derived from VEGF mRNAs produced by alternative splicing of the primary transcript (39, 40). The major band is derived from VEGF₁₆₄ mRNA.

**In Vivo Effects of Epo on Uterine Endometrium**—To examine the effects of Epo on uterine tissues in vivo and compare with those of E₂, Epo was injected into one of the uterine bilateral cavities of OVX mouse and saline into the contralateral cavity as a control. E₂ or olive oil (solvent for E₂) was given intraperitoneally. At 24 h after injection, we inspected uterine tissue sections under light microscopy. Administration of E₂ to OVX mice caused development of uterine tissues including uterine hyper trophy and endometrial growth (compare Fig. 3c with a and b). Similar development was found in the uterus after injection of Epo (Fig. 3e). Little enlargement of the uterus occurred in OVX mouse that received saline (compare Fig. 3, a and d).

A clear difference between E₂- or Epo-injected mouse and oil- or saline-injected mouse was found when endometrial sections were stained with the anti-factor VIII antibody, an endothelial cell-specific marker; blood vessel formation was stimulated upon administration of Epo (Fig. 3f) as well as E₂ (Fig. 3h). To demonstrate this stimulation by a more quantitative manner, we counted blood vessels in the sections. Stimulation by Epo was similar to that by E₂ when vessel density was expressed per field; both increased the density by 210% (Fig. 4a). However, endometrial sections from E₂-injected mouse were significantly lower in cell density than those from Epo-injected mouse (see Fig. 3, h and j). Thus, the vessel number per endometrial cell increased by 440% upon E₂ injection, whereas the increase by Epo injection was 230% (Fig. 4b). E₂ causes a rapid increase in microvascular permeability, resulting in a lower cell density of the endometrium (10). Moreover, the action of Epo somewhat differs from that of E₂ in the morphology of vessels; most of the vessels induced by Epo were smaller in diameter than those induced by E₂. Neither development of uterine tissues nor stimulation of blood vessel formation was observed when Epo was pretreated with an excess amount of sEpoR (data not shown).

**In Vivo Effects of sEpoR on Uterine Endometrium**—The cyclic remodeling of uterine tissues in the murine estrus cycle takes place every 3–5 days. The above-mentioned results suggest that Epo is involved in the estrus cycle-dependent endometrial growth through stimulation of angiogenesis. To demonstrate this possibility, sEpoR or the heat (56 °C, 30 min)-inactivated sEpoR was injected into one of the uterine bilateral cavities of the non-OVX mouse in diestrus stage and saline into the contralateral cavity. At 24 h (proestrus) after injection, we inspected uterine tissue sections under light microscopy. Transition from diestrus (Fig. 5a) to proestrus (Fig. 5d) caused endometrial growth. This endometrial growth was severely inhibited by the injection of sEpoR (Fig. 5b) but not by the inactivated sEpoR (Fig. 5c). To quantify the effect of sEpoR, the areas of myometrium and endometrium layers were calculated. The endometrium/myometrium ratios in the sEpoR-injected uterus were significantly smaller than those in the inactivated sEpoR- or saline-injected uterus (Table I), indicating that sEpoR was detrimental to the endometrial growth in the transition from diestrus to proestrus. Further detailed inspection of

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**Fig. 4. Epo- and E₂-induced endometrial angiogenesis in OVX mouse.** Sections stained with anti-factor VIII antiserum (Fig. 3, g–j) were used to count vessel density in the endometrium. a, total vessels per mm²; b, total vessels per 10⁵ cells. Shaded columns indicate the results of control mice that received injection of olive oil or saline, and black columns indicate those of mice that received injection of E₂ or Epo. Individual vessels were counted on a 19 × 19 cm monitor by color video camera. Four to six mice were used for each experiment, i.e. olive oil, E₂, saline, and Epo. More than 50 sections per uterus and at least 2 fields per section were selected at random for counting. The total number of cells was also counted per section. Results are expressed as mean ± S.E. Asterisks indicate p < 0.05, significantly different from the values of controls (oil or saline). The statistical significance of differences was determined with Student's t test.
magnified sections stained with the anti-factor VIII indicated that the formation of blood vessels in the endometrium is inhibited by sEpoR but not by the inactivated sEpoR (Fig. 5, e–g).

Expression of EpoR in the Endometrial Endothelial Cells—Immunochemical staining of the uterine section from non-OVX mice in the estrous stage using the antiserum against the extracellular domain of EpoR (Fig. 6, a and b) and anti-factor VIII antiserum (Fig. 6, c and d) showed that the uterine microvascular endothelial cells express EpoR as well as factor VIII.

DISCUSSION

We found that the uterus is a novel site for Epo production. E2 has been reported to influence Epo production (41, 42), but

FIG. 5. Injection of sEpoR into mouse uterine cavity inhibited growth of the uterine tissues, accompanied with the poor angiogenesis in the endometrium. Non-OVX 10-week-old mice in the diestrus stage received injection of test materials into their uterine cavities as described under “Experimental Procedures.” At 24 h after injection, uterine tissues were processed. a–d, transverse sections of uteri stained with hematoxylin; e–g, high power views of endometrium stained immunocytochemically with anti-factor VIII antiserum; and h–j, those of endometrium stained with the control rabbit IgG. a, diestrus uterus; b, e, and h, 8 μg of sEpoR injection; c, f, and i, 8 μg of heat-inactivated sEpoR injection; d, g, and j, saline injection. E and M indicate endometrium and myometrium, respectively. Arrows in e–g highlight typical blood vessels. Bars are 500 μm in a–d and 50 μm in e–j.

TABLE I

|                  | Injection                  | Area ratio (endometrium/myometrium) | Relative ratio |
|------------------|----------------------------|-------------------------------------|----------------|
|                  | Saline (n = 5)             | 2.03 ± 0.13                         | 1.00           |
|                  | sEpoR (n = 7)              | 1.35 ± 0.24*                        | 0.66           |
|                  | Heat-inactivated sEpoR (n = 7) | 1.81 ± 0.45                      | 0.89           |

* p < 0.05, significantly different from the values when saline or heat-inactivated sEpoR was injected. Three groups were compared using one-way analysis of variance. p was calculated using a Fisher's PLSD.

FIG. 6. EpoR is expressed in microvascular endothelial cells in endometrium. Uteri of 10-week-old mice (non-OVX) in estrus stage were used for immunocytochemical detection of EpoR and factor VIII in vascular endothelial cells. a and c, low power views of endometrium; b and d, high power views of a and c, respectively. a and b, stained with anti-EpoR antiserum; c and d, stained with anti-factor VIII antiserum. Arrows in a and c shows representative blood vessels and those in b and d indicate endothelial cells. Bars are 50 μm (a and c) and 25 μm (b and d).

the production site responding to E2 was not known. The major regulator in the uterus appears to be different from that in other Epo production sites. Oxygen is a primary signal for regulation of Epo biosynthesis in the kidney and liver; hypoxia induces transcriptional activation of the Epo gene (reviewed in Ref. 43). This induction requires de novo protein synthesis (43). In contrast, the E2-induced increase of Epo mRNA in the
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Giving Epo. Presumably the low cell density results from an much lower than that in erythroid precursor cells; the Sasaki, unpublished observations. manuscript in preparation.

E2-regulated signal molecules that is required for execution of proestrus stage, these results indicate that Epo is one of the dothelial cells. Taken together with the inhibitory effect of which has been thought to indicate a common developmental lineages between hematopoietic cells and endothelial cells (46).

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