Vascular endothelial growth factor induces mRNA expression of pro-inflammatory factors in the uterine cervix of mice

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ABSTRACT

Inflammation is believed to play a role in uterine cervical remodeling and infection-induced preterm labor. One of the distinct features of remodeling uterine cervix is presence of prominent vascular events, such as angiogenesis, vasodilation, and vascular permeability. Although the functional significance of these features is not yet clear, we know that in most tissue types, vascular remodeling is intricately intertwined with inflammation. Since vascular endothelial growth factor (VEGF) is the major architect of vascular remodeling, we sought to examine and elucidate the potential relationship between VEGF and inflammation in the uterine cervix of non-pregnant mice. The animals used were divided into 4 treatment groups: A) negative control (vehicle only), B) positive control (lipopolysaccharide, LPS), C) recombinant VEGF-164 protein, and D) LPS + VEGF blocker \( (n = 3) \). After the appropriate treatments, the uterine cervices were harvested and analyzed using real-time PCR and confocal fluorescence microscopy. Results showed that exogenous VEGF up-regulates expression of interleukin (IL)-6 and tumor necrosis factor (TNF)-α mRNAs, whereas VEGF blocker partially diminishes the LPS-induced expression of pro-inflammatory factors compared to the positive control group. We conclude that a positive feed-forward relationship likely exists between VEGF and inflammation in the uterine cervix, thus implicating VEGF in inflammation-induced preterm labor.

Inflammation is believed to play a role in uterine cervical remodeling and infection-induced preterm labor. During the course of pregnancy, the remodeling uterine cervix is, among other events, characterized by distinct vascular changes, notably, angiogenesis, vasodilation, and vascular permeability \( (7, 26–28) \). Although the functional significance of these changes during uterine cervical remodeling and at birth is, as yet, unclear, we know that vascular remodeling and inflammation are intricately intertwined \( (4) \). For instance, three of the five cardinal signs of inflammation are dependent on vascular changes, i.e., redness and heat are dependent on angiogenesis and vasodilation, whereas swelling or edema is dependent on leaky vessels or vascular permeability \( (33) \). More importantly, and of relevance to the present study, is the fact that uterine cervical remodeling and the birth process are generally considered inflammatory-like responses \( (25) \), in part, due to accumulation of immune cells and expansion of the vascular network in the uterine and uterine cervical tissues.

The extent of tissue infiltration by different immune cells varies over the course of pregnancy, during and after birth \( (4) \). Data from non-human primate model studies showing successful blockade of infection-induced preterm labor using toll-like receptor 4 antagonists appear to support the role of in-
flammation in the birth process. Other evidence is from studies using mice deficient in receptors for classical inflammatory factors, such as interleukin (IL)-1α and tumor necrosis factor (TNF)-α, or those showing that mice treated with blockers of these receptors are more resistant to the toll-like receptor agonist (lipopolysaccharide, LPS)-induced preterm birth (14, 15). It is important to note that the association of inflammation in normal uterine cervical remodeling and birth has recently been disputed by some studies (5, 40, 41). For instance, it has been shown that pharmacological depletion of inflammatory cells in the uterine cervix does not block cervical remodeling or parturition, and that although there is increased tissue infiltration by immune cells in late pregnancy, immune cells are not activated until after birth (40). Secondly, it has been shown that physical and biomechanical changes in the uterine cervix do not match collagenase activity (5). However, there is still a general consensus that preterm birth induced by infection is due to abnormal induction of the inflammatory response in the uterine cervix.

We have previously characterized the presence and profile of vascular endothelial growth factor (VEGF), the key architect of vascular events, and its key receptors in the uterine cervix of rodents (mice and rats), and delineated VEGF-related genes using DNA microarray analysis (26–28). The effects of VEGF are mediated by two tyrosine-kinase receptors, namely VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), which have different signaling properties and are largely expressed by endothelial cells (11). Other cellular targets of VEGF, that express VEGF receptors, include osteoblasts, mononuclear phagocytes, hematopoietic cells, and some malignant cells (6, 11). We have previously localized the two VEGF receptors in a number of cell types in the rodent uterine cervix (26, 27).

VEGF has been shown to induce expression of pro-inflammatory factors in a variety of tissue types, including the gut, brain, lungs, and uterus (8, 24, 34). However, to date, there is no report that directly links VEGF and inflammation in a normal uterine cervix and vice versa, other than data generated from our preliminary studies (30, 39). Because of the close association of infection, inflammation and vascular remodeling in general, and the prominence of vascular remodeling in uterine cervical remodeling, it is important that we understand how these processes interact with each other and impact the birth process.

Here, we examined the potential relationship and interaction between VEGF and inflammation in the uterine cervix of mice using VEGF agents (recombinant VEGF protein, VEGF blocker), LPS, confocal fluorescence microscopy, and real-time PCR.

MATERIALS AND METHODS

Animals used in the study. Non-pregnant ovariectomized and pregnant (day 11 and 17) mice (C57BL6/129SvEv, from Charles Rivers) were used in the present study. Prior to treatments, all non-pregnant animals were ovariectomized under general anesthesia using a mixture of ketamine (43–129 mg/kg body wt) and xylazine (8.6–26 mg/kg body wt), followed immediately after surgery by administration of Baytril® antibiotic (Bayer, Leverkusen, Germany), to prevent post-surgery infections. Animals were allowed to rest for at least seven days prior to treatments, to allow complete excretion of residual ovarian sex steroid hormones. Before tissue harvest, animals were euthanized by lethal injections of Sleepaway® (concentrated sodium pentobarbital; Fort Dodge Laboratories Inc., Burlingame, CA). Uterine cervical tissues were harvested, processed and analyzed, following appropriate treatments. Harvested tissues were analyzed using morphological techniques (confocal fluorescence microscopy) and gene expression (real-time PCR), in order to elucidate the effects of VEGF on uterine cervical inflammation and its relationship or interaction with infection-induced or lipopolysaccharide (LPS)-induced inflammation. All procedures were performed in accordance with the regulations established by the Appalachian State University Animal Care and Use Committee, the Guide for the Care and Use of Laboratory Animals of our local institution, and NIH Publication No. 86–23. In an effort to minimize the number of animals used in these experiments, the sample sizes per treatment for optimization studies were limited to an n = 1. However, subsequent studies had at least an n = 3.

Treatment with exogenous VEGF. For determining the optimal dosage of VEGF, mice were divided into 3 treatment groups, dose-dependently (n = 1): 1) Group I, the negative control, was only administered the vehicle, i.e., 25 μL of saturated (25%) Pluronic F-127 (Sigma Aldrich, St. Louis, MO) in 0.1 M PBS, intra-vaginally (IV); 2) Group 2 was treated with 20 ng of recombinant VEGF (Calbiochem, La Jolla, CA), dissolved in the same vehicle, IV; 3) Group 3 was treated with 200 ng of recombinant VEGF. Each group was treated with 2 consecu-
tive injections at time = 0 h and time = 4 h and all tissues were harvested at time = 8 h post-initial VEGF treatment and processed for analysis. Optimal administrative routes for VEGF were also investigated by comparing intraperitoneal (IP) and intra-vaginal (IV) routes and combinations of the two routes.

For determining optimal duration and number of VEGF treatments, we examined when mRNA levels of pro-inflammatory cytokines were at their greatest after a single and repeat (twice) VEGF treatments using the optimal treatment conditions (dosage, frequency, and route), determined earlier in the section above. In single treatments, mice were divided into 6 treatment groups, based on the time of tissue harvest post-VEGF injection (n = 1), namely time = 1, 2, 4, 6, 8, and 10 h. Animals were handled and tissues were harvested, as described earlier, and processed for mRNA levels of pro-inflammatory markers (IL-6 and TNF-α) using real-time PCR. In repeat treatments, mice were divided into 10 treatment groups (n = 1), based on time after initial VEGF injection, i.e., time = 0, 1, 2, 4, 5, 6, 8, 10, 12, and 14 h. Mice were treated with two consecutive injections of recombinant VEGF, in the same manner as described above with time of VEGF administration = 0 h and 4 h, respectively. Uterine cervical tissues were harvested between 5–14 h post-initial VEGF injections and processed for mRNA levels of pro-inflammatory markers (IL-6 and TNF-α) using real-time PCR (see qRT-PCR below). We also performed experiments that examined the effect of repeat treatments of VEGF on the expression of its receptors, Flt-1 and KDR.

**VEGF tracking studies.** First, the most effective carrier for VEGF uptake across uterine cervical epithelia via intra-vaginal administration was determined using robustness in mRNA expression of pro-inflammatory factors. Mice were divided into 3 treatment groups (n = 1): 1) Group 1 was negative control and was only administered PBS (50 μL of 0.1 M PBS, IP); 2) In Group 2 methycellulose was used as a carrier (200 ng VEGF dissolved in 25 μL of 2% methycellulose; MP Biomedicals, Solon, OH) and 0.1 M PBS, IV; and 3) in Group 3 pluronic gel was used as the carrier (200 ng VEGF dissolved in 25 μL of saturated pluronic gel and 0.1 M PBS, IV). Each group was treated with 2 consecutive injections at time = 0 h and time = 4 h, and all tissues were harvested at time = 8 h post-initial treatment.

Prior to the VEGF tissue tracking study, VEGF was tagged with fluorescence and the degree of label (DOL) was estimated. An Alexa Fluor® 488 (in-vitrogen) labeling kit was used to tag VEGF. An amount of 20 μg VEGF protein was dissolved in 20 μL of 1 M sodium bicarbonate buffer to make a 1 mg/mL solution. A 2.77 μL reactive TPF ester was added to the protein mixture. After allowing 15 min for protein-dye conjugation, a spin filter was used to separate the labeled VEGF protein from unreacted dye. Absorbance at 494 nm was used to quantify the amount of Alexa Fluor® 488 attached to VEGF with a Shimadzu UV 2401 PC double beam ultra-violet visible spectrophotometer. Using this absorbance and the protein concentration, the DOL was estimated to be 10.

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DOL = \frac{\text{Alexa Fluor® absorbance max} \times \text{dilution factor}}{\text{Molar absorptivity of Alexa Fluor®} \times \text{VEGF concentration}}
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For visually tracking the movement of VEGF tagged with Alexa Fluor® 488, mice were divided into 4 timed-treatment groups, i.e., time = 10 min, 30 min, 60 min, and 120 min post-treatment (n = 1), and then treated with a single injection of fluorescent-tagged VEGF (120 ng dissolved in 25 μL of saturated pluronic gel and 0.1 M PBS, IV). Animals were given a lethal injection of Sleepaway® (50 μL), and uterine cervical tissues were immediately harvested, cryostat-sectioned at 20 μm and processed for confocal microscope imaging.

**Treatment with LPS.** In the initial experiments of this study, we optimized the treatment conditions for LPS, i.e., dosage and optimal duration for maximal expression of pro-inflammatory factors. Optimal conditions were determined by IL-6 mRNA levels using real-time PCR and were utilized in subsequent experiments that investigated the relationship between VEGF and LPS. In this experiment, we sought to delineate VEGF-mediated inflammation in LPS-treated animals using IL-6 mRNA as a marker. The animals were pre-treated with a VEGF blocker [PTK 787/ZK22584, (1-[4-chloroanilino]-4-[4-pyridylmethyl] phthalazine succinate]; a generous gift from Novartis Pharma AG, Basel, Switzerland] prior to LPS administration. Mice were divided into 4 treatment groups (n = 3), as follows: 1) Group 1 was given two consecutive injections of recombinant VEGF (dosage and route as described above, under VEGF optimization) administered at time = 0 h and 4 h and tissues were harvested at time = 8 h; 2) Group 2 was given a single LPS injection (100 μg dissolved in 50 μL of 0.1 M PBS, IP) and tissues were harvested 1 h later; 3) Group 3 was initially given a single injection of VEGF blocker, followed 1 h later by a single LPS injection, i.e., 5 mg VEGF.
Data were analyzed using Student's *t*-test and ANOVA, followed by Scheffe's *F*-test for multiple comparisons. *P*-values of < 0.05 were considered to be statistically significant.

**RESULTS**

The optimal conditions for effective delivery of recombinant VEGF were as follows: 2 consecutive injections of 200 ng of VEGF dissolved in 25 μL saturated (25%) pluronic gel/0.1 M PBS, delivered intra-vaginally (IV) at time = 0 h and time = 4 h. Tissues were harvested at time = 8 h (Figs. 1–3). With a single injection of VEGF, the most effective dosage in inducing the greatest inflammatory response, based on uterine cervical IL-6 mRNA expression, was 200 ng (27-fold greater than negative control) compared to 20 ng (5-fold greater than negative control) (Fig. 1A).

Studies determining the duration of VEGF’s optimal inflammatory response (IV administration) revealed that an hour after a single VEGF injection, the first increase in TNF-α mRNA expression was observed, reaching peak levels around 2 h (5-fold greater than negative control), and returning to baseline at about 8 h (Fig. 1B). The first increase in IL-6 mRNA expression, following the single injection of VEGF, was seen around 2 h post-treatment (Fig. 1B) and peaked around 4 h (34-fold greater than negative control), returning to baseline at about 6 h (Fig. 1B). However, the initial increase in IL-6 was more pronounced than TNF-α (Fig. 1B). Repeat (twice) treatments of VEGF generated a more robust inflammatory response than the single treatment, *i.e.*, an hour after the second VEGF injection, TNF-α mRNA expression increased to its peak levels around time = 5 h (97-fold greater than negative control) and progressively decreased to only 10-fold greater than negative control by the end of the experiment, time = 14 h (Fig. 1C). The highest increase in IL-6 mRNA expression was seen at time = 8 h, later than the first increase in TNF-α mRNA expression (Fig. 1C). IL-6 mRNA levels peaked around 8 h after the first VEGF injection (600-fold greater than negative control), steeply declining by 10 h (34-fold greater than negative control), and slightly increased in expression until the end of the experiment (70-fold greater than negative control at time = 14 h) (Fig. 1C). Repeat treatments had no effect on the transcription of VEGF receptors (Flt-1 and KDR) (data not shown).

Administration of VEGF using saturated pluronic gel and 0.1 M PBS as the carrier vehicle elicited the most robust expression of IL-6 mRNA (37-fold greater than negative control) compared to 2% methylcellulose-treated group (10-fold greater than negative control) (Fig. 2A). Tissue uptake and tracking studies detected the administered recombinant VEGF in uterine cervical epithelial tissue as early as 10 min. Between the 30 and 60 min tissue collection times, there was increased fluorescence inside the uterine cervical tissue (Fig. 2B). The infiltration of VEGF into the tissues from 10–60 min increased in a time-dependent manner (Fig. 2B). However, between 60 and 120 min, there was less fluorescence illumination from the tissues (Fig. 2B).

For studies designed to examine the relationship between VEGF and inflammation, the greatest up-regulation of IL-6 mRNA expression was found in LPS-treated mice (9,000-fold greater than negative control), which served as a positive control (Fig. 3). VEGF blocker inhibited LPS-induced pro-inflammatory mRNA expression by 2,900-fold (Fig. 3). Mice treated only with VEGF showed up-regulated expression of IL-6 mRNA (76-fold greater than negative control) (Fig. 3). The difference between the pluronic gel group and VEGF blocker + LPS group was statistically significant (*P* = 0.024).

TNF-α protein was localized primarily in uterine cervical epithelial cells and was also expressed by a variety of other cells types in the uterine cervical tissue matrix, which are most likely stromal, immune or vascular cells (data not shown).

**DISCUSSION**

The purpose of the present study was to examine the existence of a potential interrelationship between VEGF and inflammation in the uterine cervix. The key findings from this study are: 1) VEGF induces expression of classical pro-inflammatory factors in mouse uterine cervix in a dose-dependent manner; 2) Pluronic gel is more effective in mediating trans-epithelial uptake and delivery of exogenous VEGF protein into the uterine cervix compared to methylcellulose; 3) Repeat intra-vaginal administration of VEGF induces a more robust inflammatory response than a single treatment; and 4) LPS-induced inflammatory response is, in part, mediated by VEGF. This study establishes that VEGF induces inflammation...
VEGF and inflammation in uterine cervix

The optimal dosage of VEGF used in the present study is within the physiological levels found in the uterine cervix of pregnant rats at day 22, as determined by ELISA in our previous study (2), implying that this optimal dosage is physiologically relevant and endogenous VEGF may exert the inflammatory effects in non-pregnant animals observed here. However, it must be noted that non-pregnant ovariectomized mice were used in the present study, whereas pregnant rats were used in our earlier studies (27). Further, the mean VEGF concentrations obtained in human studies, i.e., 282 pg/mL (10) or 184 pg/mL (serum) (19) were significantly higher than mouse levels used in the present study.

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**Fig. 1** Optimization of VEGF treatment in mice uterine cervix, as revealed by real-time PCR (qRT-PCR). A) Dosage studies (IL-6 mRNA expression): Animals were treated with different dosages of recombinant VEGF/25 μL pluronic gel, IV, and tissues were harvested after 8 h. Injecting 200 ng/25 μL showed the greatest up-regulating effect on IL-6 mRNA levels, determined to be the optimal VEGF dosage. B) Time-dependent studies using single treatments. Tissues were analyzed for IL-6 and TNF-α mRNA expression. Animals were treated with 200 ng VEGF/25 μL pluronic gel, IV. Tissue harvested at 4 h after injection had the greatest up-regulating effect on levels of IL-6 mRNA and tissues harvested 2 h after injection had the greatest up-regulating effect on TNF-α mRNA levels. C) Repeat treatments: Animals were treated with VEGF protein, IV at time = 0 h, given a booster at time = 4 h, and tissues were harvested at different times after the 2nd injection. The greatest up-regulation of IL-6 mRNA levels was at 4 h after a 2nd VEGF injection. The greatest up-regulation of TNF-α mRNA levels was at 1 h after a 2nd VEGF injection. Time = 0 h on graph represents negative control, given no VEGF treatment.
ly, the fact that administration of a VEGF blocker diminished expression of LPS-induced inflammatory markers, albeit slightly, suggests a role for VEGF in infection (LPS)-induced preterm uterine cervical remodeling. This observation is consistent with our recent studies that showed that LPS also increased levels of VEGF and its receptors in the uterine cervix of non-pregnant ovariectomized rodents (30, 39) and, in turn, attenuation of VEGF action via use of VEGF blocker sharply decreased mRNA expression of LPS binding protein and an array of inflammatory factors in uterine cervix (28). Taken together, these data may suggest existence of a positive feed-forward loop between the vascular and inflammatory factors in the uterine cervix and implicates aberrant VEGF expression patterns in the patho-

**Fig. 2** Optimal carrier and tissue tracking of exogenous VEGF in mice uterine cervix and detection of fluorescence-tagged VEGF. **A)** Optimal carrier: Animals were treated with 200 ng recombinant VEGF/25 μL of either cellulose or pluronic gel, IV, at time = 0 h, again at time = 4 h, and tissues were harvested at time = 8 h. Injecting pluronic gel showed the greatest up-regulating effect on IL-6 mRNA levels, determined to be the optimal injecting vehicle. Negative control was given no treatment. **B)** Fig. 2B shows a time-dependent movement of fluorescent-tagged recombinant VEGF in mouse uterine cervix using confocal microscopy. L = lumen; e = uterine cervical epithelial cells.

Although the present study is the first to directly demonstrate VEGF’s stimulatory effects on the expression of pro-inflammatory factors in the uterine cervix, this phenomenon is widely reported in a variety of other tissues. For instance, VEGF from myeloma cells induced IL-6 secretion from microvascular endothelial cells and bone marrow stromal cells, associated with multiple myelomas (9, 23). Also, Yoo *et al.* (43) showed that VEGF directly increased the production of IL-6 from peripheral blood mononuclear cells and was likely involved in the pathogenesis of rheumatoid arthritis. Ongoing studies in our lab are investigating whether VEGF will also induce an increase in the protein levels of inflammatory factors and whether this phenomenon is physiologically relevant during pregnancy.
VEGF and inflammation in uterine cervix

The role of inflammatory factors in preterm labor is well established (12, 13, 31, 32), with infection-induced preterm labor accounting for the bulk of preterm labor, i.e., about 25–40% (20). Animal studies have shown that mice with deletions in both IL-1 and TNF-α receptors have a significantly reduced susceptibility to bacteria-induced preterm labor (14). We also know that the inflammatory signaling pathway is extensive and is notoriously redundant. For instance, although deletion of IL-1 receptor in knockout mice reduces susceptibility of bacteria-induced preterm labor, IL-1 signaling is not required in bacteria-induced preterm labor when the TNF signaling pathway is intact (14). Furthermore, Holmgren et al. (15) showed that although administration of TNF-α antagonist prior to injection of an inflammatory mediator (LPS) could significantly decrease preterm birth in mice, administration of anti-TNF-α did not completely eradicate the effects of LPS. These data indicate that TNF-α plays an important but non-obligatory role in LPS-induced preterm labor and that there are other factors, other than TNF-α, that may have a positive feed-forward effect on TNF-α, perhaps, such as VEGF (15). More studies need to be conducted that will shed more light on VEGF’s exact role in this extensive inflammatory signaling pathway of the remodeling cervix.

Various carriers have been used and developed to facilitate the uptake of proteins across cell membranes (16, 37). In this study, pluronic gel (a nano-container) was compared to methylcellulose. We found that pluronic gel facilitated a greater uptake of fluorescence-tagged exogenous VEGF across the uterine cervical epithelium than methylcellulose. The tracking data clearly shows the fate of the fluorescent-tagged VEGF protein as it is delivered into the uterine cervical tissue in a time-dependent manner. The decrease in VEGF detection by 120 min could possibly indicate degradation of VEGF protein by local protease enzymes in the uterine cervix, which is overall consistent with the timeline of the maximal effect of VEGF on the mRNA expression of inflammatory factors.

Various studies have previously utilized blockers and agonists for inflammatory and vascular factors in order to better understand their interactions. For instance, Valpha, a decoy receptor that binds both VEGF-A and TNF-α blocks both angiogenesis and inflammation and has therapeutic potential for treating retinopathy and psoriasis and possibly other diseases caused by pathological inflammatory angiogenesis involving VEGF-A and TNF-α (17). On the other hand, VEGF-Trap, a blocker of VEGF action, inhibits tumor and ocular angiogenesis and reduces vascular leakage (17). Finally, Enbrel, a decoy receptor that blocks the inflammatory effects of TNF-α, has been valuable in understanding mechanisms that may underlie inflammation and vascular remodeling (17).

Fig. 3 Effects of recombinant VEGF on IL-6 mRNA expression in mice uterine cervix. LPS has an up-regulating effect on IL-6 mRNA levels compared to VEGF and negative control. VEGF blocker showed a decrease in mRNA levels compared to LPS. Negative control (vehicle only, pluronic gel, IV), positive control (LPS, 100 μg/mouse, IP), VEGF (200 ng/25 μL for 2 doses, IV), VEGF blocker + LPS (5 mg/kg VB, IP; 100 μg LPS/mouse, IP), P = 0.024 for negative control group and VEGF blocker + LPS; no other statistical significance between groups was found. n = 3.
VEGF during uterine cervical remodeling. Although the exact role of VEGF and hypoxia in parturition is not completely clear, it is interesting to note that women in Peru, when close to their due date, can intentionally induce the birth process by going to higher attitudes, where oxygen levels are low or conditions are relatively hypoxic (3). It should be noted that although chronic hypoxia in an experimental animal model induced measurable changes in maternal serum levels of VEGF and tensile properties of the rat uterine cervix, and hypoxia decreases the uterine cervical strength to stretch and predisposes to rupture, this effect appears to be unrelated to maternal serum levels of VEGF (38). There are currently ongoing studies in our lab that are investigating the role of exogenous sex steroids and acute cobalt chloride-induced hypoxia on VEGF expression in uterine cervical tissue.

In conclusion, we show, for the first time, that VEGF induces inflammation in the uterine cervix of mice in a dose-dependent manner and that VEGF may mediate some of the inflammatory effects in-
duced by LPS in a positive feed-forward manner (see proposed model in Fig. 4). These data are novel and important in that they shed new insights into the possible role of VEGF in infection-induced preterm labor, specifically uterine cervical remodeling.

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