VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS EFFECT ON THE FLUID DYNAMIC AND EPITHELIA OF THE CERVIX

A Thesis
By
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VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS EFFECT ON THE FLUID
DYNAMIC AND EPITHELIA OF THE CERVIX
(May 2010)

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Cervical remodeling (CR) is a complex process, among other things, associated with collagen dissociation, increase in edema and tissue mass, and is loosely categorized in four overlapping, but uniquely regulated stages. Our knowledge on the role of the microvasculature and the underlying mechanisms in this process (CR) is incomplete. VEGF, a potent vascular permeability factor, mitogen and key angiogenic architect, has been shown to mediate edema and cellular proliferation in several tissue types. Our lab has previously characterized expression of VEGF and its receptors in the cervix, and identified VEGF-regulated genes during CR using DNA microarray. Here, we use various techniques, serum protein tracking dye (Evans Blue), VEGF agents and rodents and show that VEGF likely plays a role in CR, in part, by inducing expression of tight junction genes, vascular permeability, serum protein tissue infiltration, edema and epithelial cell growth.

ACKNOWLEDGMENTS

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Introduction

Events in the uterus and cervix are coordinated in a highly complex and tightly regulated fashion during pregnancy and labor (Leach & Firth, 1995). One of the functions of the cervix is to act as a barrier, i.e., it prevents microbial invasion of the developing fetus and premature passage or birth of the fetus. As the fetus develops and pregnancy advances, the cervix undergoes structural changes, i.e., cervical remodeling (Leppert & Yu, 1994). Cervical remodeling (CR) is progressive and leads to softening, ripening, dilation and, ultimately, to fetal passage. During CR, but prior to dilation and uterine contraction, the cervix maintains a mechanical resistance to the gravitational forces exerted by the growing fetus. The cervix is believed to accomplish this by increasing its size and tissue volume or edema (Leach & Firth, 1995). Once the cervix has completely ripened it relaxes, dilates and, as the uterus contracts during labor, facilitates the timely passage of the fetus at parturition (Kelly, 2002). Failure of either or both of these events, lead to birthing problems such as preterm birth and protracted labor, which account for 75% of fetal mortality (Challis, 2000).

Although significant strides have been made in understanding mechanisms that underlie CR, the exact factors that trigger CR are not fully understood. Of interest to the present study is the investigation of CR-associated events, such as local vascular alterations (vascular permeability, angiogenesis and vasodilation), edema and cervical epithelial proliferation or changes in tissue mass (Collins, Wilson, Fischer-Colbrie & Papka, 2000; Mowa et al., 2004).

In particular, very little is known about the role of local microvascular alterations, the factors that regulate them and the relationships, if any, between tissue edema and local cervical vascular changes. Some of the proposed regulators of cervical microvasculature include sensory neuronal-derived factors, such as Substance P (SP). Collins et al., (2000) observed that SP in rats induced microvascular changes, such as increased vascular permeability, increased white blood cells (WBC) infiltration and vasodilatation. We have previously demonstrated that bilateral neurectomy of SP-producing neurons innervating the cervix down-regulated levels of vascular endothelial growth factor (VEGF). To date, VEGF is the best studied and key regulator of microvascular remodeling in several body tissue types. Based on these findings and the fact that VEGF plays a key role in microvascular remodeling in most tissue types, we believe that SP likely induces vascular changes in the cervix via the up regulation of VEGF levels, and hypothesized that VEGF is one of the regulators of local cervical vascular remodeling. In our subsequent studies we characterized the presence and expression patterns of VEGF isoforms, its receptors and signaling molecules, delineated VEGF-related genes by screening 30,000 genes in the cervix using DNA microarray analysis and other techniques (Mowa et al., 2004; Mowa et al., 2008). Our findings are consistent with the preliminary study that showed that VEGF alters the biomechanical properties of the cervix by diminishing tensile strength (Buhimschi, Dussably, Buhimschi, Ahmed & Weiner, 2004).

VEGF is a chemokine produced by cells such as fibroblasts. It is best known for its induction of new blood vessel formation by stimulating endothelial cell growth during embryonic development, after injury and blockage of blood vessels. VEGF is a member of a family of closely related growth factors that include VEGF-A, -B, -C, -D, -E and placenta

growth factor (PIGF). VEGF-A has well-established biological effects and exists as several

splice variants (Ferrara & Davis-Smyth, 1997). Biological effects of VEGF are largely

mediated by two receptors: KDR (kinase domain region) and Flt-1 (fms-like tyrosine kinase-

1) (De Vries et al., 1992; Ferrara & Davis-Smyth, 1997; Mustonen & Alitalo, 1995; Shibuya,

1995; Shibuya, Ito & Claesson-Welsh, 1999; Ozaki, Iwasa & Mitani, 1999). To date, VEGF

is the most potent endogenous inducer of vascular permeability ever known and is more

potent than histamine and bradykinin, i.e., about 50,000 times more potent than histamine

(Krzysztof et al., 2000; Dvorak, 2002). Other than inducing endothelial cell growth, VEGF is

also reported to induce growth of other cell types, including neuronal tissue and Schwann

cells (Rosenstein & Krum, 2004). Thus, in the present study, we sought to investigate the

effects of VEGF on local cervical vascular leakage, as well as its effects on cervical epithelial

tissue mass.

VEGF has been shown to induce vascular permeability by altering the cellular junctions

of endothelial cells, e.g., by creating fenestrae through which intravascular solutes and fluids

can infiltrate the tissue (Albrecht et al., 2003; Stan, 2007). Epithelial and endothelial cells in

several tissue types have tight junctions (TJ) that regulate passage of molecules, ions and

water through the paracellular spaces (Gonzalez-Mariscal, Avila-Flores & Betanzos, 2001).

To date, two components of the TJ have been identified, namely occludin and claudin. The

latter is a protein family consisting of over 20 members. Variations in or extent of tightness

of TJ can differ, depending on the composition and combination of claudin species involved.

Our interest in TJ is based on the fact that they are known to have a role in vascular

permeability and that they are regulated in pregnancy (Timmons, Mitchell, Gilpin &

Mahendroo, 2007). Furthermore, and of interest to the present study, claudin expression

profile exhibits a temporal and spatial relationship with VEGF during pregnancy (Mowa et al., 2004,Timmons et al., 2007) and in other tissue types, such as the fetal lung. Further, VEGF induces rapid phosphorylation of TJ proteins, occludin and zona occluden-1(ZO-1) (Antonetti, Barber, Hollinger, Wolpert & Gardener, 1999). Other studies have also shown that VEGF increases bovine monolayer endothelial cells (BMEC) permeability by altering expression of occludin and TJ assembly (Wang, Dentler & Borchardt, 2001).   We are also interested in whether VEGF alters TJ expression in the cervical epithelial cells.

Increase in cervical tissue mass during pregnancy is another obvious alteration that occurs during CR, and factors such as relaxin have been implicated in this process (Burger & Sherwood, 1995). We have speculated in the past that VEGF likely plays a role in this process by regulating events in local cervical vascular endothelial and or epithelial cells. Based on its effects on other tissue types, we have suggested that VEGF exerts its effect on the epithelial cell types (cervical epithelia and vascular endothelia) via two mechanisms: 1) VEGF may stimulate endothelial cells to secrete growth factors (FGF, IGF, PDGF) that, in turn, stimulate proliferation of neighboring epithelial cells (Mowa et al., 2004; Mowa et al., 2008), or 2) VEGF may induce vascular permeability that may lead to increase in infiltration of local tissue and induction of epithelial growth by serum factors, which are a rich source of growth factors (Tomanek & Schattman, 2000). These mechanisms may both be operational in the cervix, and may, in part, account for the phenomenal epithelial proliferation during CR, perhaps in collaboration with factors such as relaxin. Indeed, our preliminary data showed pronounced effects of VEGF on cervical epithelial folds in ovariectomized rats treated with VEGF compared to the control group (Mowa et al., 2008). It is important to note

that epithelial cell proliferation during pregnancy account for about 75% of the entire cervix and that these cell types are believed to play a key role in CR (Burger & Sherwood, 1998).

For instance, it has been suggested recently that cervical epithelial cells are involved in: 1) maintaining fluid balance via synthesis of hydrophilic hyaluronan and glycosaminoglycan and aquaporins, 2) proliferation and differentiation, 3) regulation of paracellular transport of solutes via tight junctions regulated by apical TJ proteins, 4) providing a protective barrier against invading micro-organisms, and mediating inflammatory and adaptive immune responses, and 5) acting as an "endocrine" gland by synthesizing prostaglandins, chemokines and cytokines and steroid hormones (Mitic & Van Itallie, 2001; Morita, Sasaki, Fujimoto, Furuse & Tsukita,1999a; Morita, Sasaki, Furuse & Tsukita, 1999b; Demaio, Chang, Gardner, Tarbell & Antonetti, 2001; Minijarez, Millar, Lindquist, Anderson & Word, 2000; Gonzalez-Mariscal, Betanzos, Nava & Jaramillo, 2003). Thus, in view of these observations and our recent data, it is likely that VEGF potentially impacts CR in multiple ways. Here, we focus on investigating the effects of VEGF on cervical microvascular permeability, edema and cervical epithelial proliferation using rodents, Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), basic histology, Evans Blue dyes (EBD), and real-time PCR.

Materials and Methods

Overview of Animals and Treatments

Timed-pregnant and –postpartum, as well as ovariectomized Sprague Dawley rats (SASCO strain) and C57BL6/129SvEv mice (Charles Rivers) were used in these studies (*n*=5-10 per time-point), as specified below: Uterine cervical tissues were harvested and analyzed, following appropriate treatments with either vehicle (0.1 M PBS mixed with saturated Pluronic gel under ice) or VEGF agents [mouse recombinant VEGF 164 protein (Calbiochem, La Jolla, CA), VEGF inhibitor (PTK 787/ZK22584; generously provided by Novartis Pharma AG, Basel Switzerland)], using basic histology (H&E), TEM, SEM, Evans Blue dye (vascular leakage), edema and real-time PCR (gene expression). The effects of VEGF on cervical vasculature, particularly microvascular permeability and edema were investigated. Rats were used in the vascular permeability, edema and SEM studies due to their larger cervical tissue volume, while mice were used in the remainder of the studies, namely histology, TEM and real-time PCR. Prior to tissue harvest, animals were euthanized with Sodium Pentobarbital (Sleepaway**®,** Fort Dodge Laboratories Inc., Burlingame, CA). For surgeries, animals were anaesthetized with ketamine and xylazine (43-129 mg Ketamine and 8.6-26 mg Xylazine/g of body weight), ovariectomized and then allowed to rest for seven days before performing the experiments.

All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the local institution and the NIH guidelines (NIH publication number 86-23), to minimize both animal suffering and numbers of animals used.

Determination of VEGF's effects on Cervical Vascular Leakage using Evans Blue Dye

This experiment was undertaken in order to determine the effects of VEGF on local cervical vascular permeability. Ovariectomized Sprague Dawley rats were randomly separated into two treatment groups (n=9). A 1% solution of EBD (Sigma Aldritch, International) in 0.1 M Phosphate Buffered Saline (PBS) (pH 7.5) was initially sterilized using 0.22 µm pore filters (Pall Corporation, Ann Arbor, MI) and then was injected intraperitoneally (i.p.), 20 hours prior to tissue collection. The volume of EBD solution administered was 1% of body mass (1mg EBD/0.1ml PBS/10 g body mass). Six hours later, the animals were then treated with either 50 µl of VEGF agents or vehicle, as described under the edema section (see below). Tissues were harvested 20 hours post-EBD injection and 14 hours post treatment, then immediately placed in formamide for 24 hours in order to extract EBD. The optical density of the formamide solution was then measured using a spectrophotometer (Beckman DU 640B, Analytical Instruments, LLC, Golden Valley, MN) at 620 nm, in order to determine the concentration of EBD in the tissue, based on values from a standard curve.

Determination of VEGF's Effects on Cervical Edema

This experiment was performed in order to determine whether VEGF-induced vascular permeability will lead to edema formation. Animals were divided into three treatment groups as follow: 1) mouse recombinant VEGF 164 protein once daily for four

days; 2) VEGF receptor antagonist (5 mg/rat once daily four days) or 3) vehicle only (50 µl once daily). For the VEGF protein-treated animals, dose response experiments were performed in order to determine an optimal dose, based on drug delivery route (intravenously or intraperitoneally, i.v. or i.p., respectively) and dosage (10, 20, 40 ng). Animals were treated once daily for four days. Forty (40) ng (i.p., route) was determined to be the optimal dose and route, respectively, based on the magnitude or degree of tissue edema and gene expression, and was used in subsequent studies, as described earlier. Tissues were harvested six hours post-treatment, weighed wet, and snap frozen in liquid nitrogen. Tissues were then lyophilized by placing them under vacuum in a speed vac (Freeze dry system/Freeze zone 4.5, Labco, Kansas City, MO) at –40 C for 24 hours. Finally, the tissues were weighed (dry) again to determine the dry weight. Tissue edema was determined as a percentage of dry weight over wet weights.

Determination of VEGF's effects on Expression of Cervical Inter-epithelial Cell TJ mRNAs

Gene expression studies were performed on animals treated as described above (n=5) to determine mechanisms that may underlie VEGF-induced cervical inter-epithelial cell permiability in mice using real-time PCR in order to complement the morphological and functional studies discussed above.

Tissue processing, messenger RNA isolation and quantification. The animals were euthanized and transcardially perfused with normal saline. The cervices were removed and stored at -80 °C until processing. Total RNA was isolated from cervices of individual animals using RNeasy Mini Kit (Qiagen, Valencia, CA). The amount and purity of total RNA for each sample was estimated by spectrophotometric analysis at A260 and A280.

The quality of RNA was determined by agarose gel electrophoresis following SYBR green™ (Invitrogen) staining. Aliquots of total RNA were diluted in diethylpyrocarbonated (DEPC)-treated water and stored at -80 °C.

Reverse transcriptase PCR. Total RNA from tissues was reverse transcribed and amplified in an Eppendorf Master Cycler, using reagents from Gene AMP Gold RNA PCR Kit (Perkin Elmer BioSystems, Foster City, CA). The RNA was used in RT-PCR to evaluate the total levels of the genes of interest, as described under real-time PCR.

Real-time PCR. The cDNA generated above was used to evaluate the relative expression of select genes with fold change of equal or greater than 2, including TJs (occluden, claudin-1 and -2) and cell adhesion molecules (E-selectin, and VCAM-1) using specific TaqMan Gene Expression Assays (Applied Biosystems, Foster, CA), which are pre-designed and pre-optimized gene-specific probe sets. DNA amplification was performed using the Applied Biosystems (ABI 7300 HT) Real-Time PCR machine with the GeneAmp 7300 HT Sequence detection system software (Perkin-Elmer Corp). The PCR reactions were set up in wells of a 96-well plate in a volume of 25 μl per well. The reaction components were: 50 ng (5.0 μl) of synthesized cDNA; 12.5 μl of 2×TaqMan Universal PCR Mastermix; 1.25 μl of 20×Assays-on-demand™ Gene Mix (e.g., VCAM-1); and 6.25 μl of RNase-free water. The program was set as follows: an initial step at 95 °C for 10 minutes, and then 40 cycles of 95 °C for 15 seconds, and 60 °C for 60 seconds. The relative amount was calculated from the threshold cycles with the instrument's software (SDS 2.0), according to the manufacturer's instructions. Relative expression levels of the target genes were normalized to the geometric mean of the internal control gene, GAPDH. *P*-values of < 0.05 were considered to be statistically significant.

Determination of VEGF's effects on Cervical Epithelial growth using SEM and Basic Histology

SEM experiments were undertaken to visualize, in more depth, the effects of VEGF agents on the morphology and proliferation of cervical epithelia, reported in our earlier studies (Mowa et al., 2008). Ovariectomized Sprague Dawley rats were divided into three groups (n=3) and treated intra-vaginally with 50 µl of either: 1) mouse recombinant VEGF 164 protein (40 ng/day/5 days), 2) VEGF receptor antagonist (5 mg /day/5 days) followed by mouse recombinant VEGF 164 protein (40 ng/day/5 days) after 6 hours, or 3) vehicle (50 µl/day/5 days). Tissues were harvested and fixed in 2.5% glutaraldehyde in 0.1 M PBS immediately after sampling. After overnight fixation, the tissues were washed with the buffer, then dehydrated in a graded series of ethanol, and dried with a Polaron critical point drying apparatus (Polaron Instruments Inc., Doylestown, PA). All dried samples were mounted on aluminum stubs, sputter coated with gold, and imaged with a Quanta 200 SEM (FEI Company, Hilsboro, OR) at 20 kV.

Basic histology experiments were undertaken to complement the morphological and functional data generated under edema and vascular permeability studies. Briefly, ovariectomized animals were divided into three treatment groups, as described earlier under the edema section (n=25). An hour after the last treatment on day 5, the animals were euthanized and then perfused with normal saline (0.9% sodium chloride). The cervical tissues were harvested and placed in 4% paraformaldehyde for fixation. The tissues were then dehydrated, immersed in xylene and infiltrated with paraffin using a Thermo Scientific Shandon Citadel 1000 tissue processor as follows: **1)** *Dehydration*: 70% ethanol overnight; 80% ethanol for 30 minutes., 95% ethanol for 30 minutes., 100% ethanol for 40 minutes,

and another round of 100% ethanol for 40 minutes. Then the pre-waxing treatment begins. **2)** *Pre-waxing treatment*: 100% Xylene for two 40 minutes cycles in fresh solutions, **3)** *Waxing*: Paraffin bath 1 for 40 minutes; and paraffin bath 2 for 60 minutes, followed by paraffin embedment and overnight curing in a transverse orientation. After blocks were cured and hardened, they were then sectioned at 7 µm using a microtome (Reichert Jung Bio-cut 2030, Reichert, Depew, NY) then stained with H&E, according to the manufacturer's instruction, and viewed on an inverted Zeiss DIC light microscope. The average height of cervical epithelial (30 cells) were obtained using the software MicroSuite Five (2009) for each treatment group and plotted as a graph, to determine the effects of treatments on epithelial height or growth.

Determination of VEGF's effects on Cervical Epithelial Paracellular Space using TEM

TEM experiments were performed in order to localize sites and appearances of paracellular spaces (perforations) between cervical epithelial cells, following administration of VEGF. This study (TEM) complemented the basic histological and SEM studies. Briefly, timed pregnant mice [13-20 gestation day (GD)] were divided into four groups based on treatment (n = 5/treatment). These animals were treated intra-vaginally with 25 µl of: **1)** mouse recombinant VEGF 164 Protein (40 ng/mouse once daily from GD 13-17), **2)** VEGF receptor antagonist (5 mg/mouse once daily from GD 13-17), **3)** VEGF receptor antagonist, followed 6 hours later with mouse VEGF recombinant 164 protein (as described earlier) or **4)** vehicle, following a similar treatment regimen, as described earlier for treatment groups. VEGF receptor antagonist was pre-dissolved in DMSO then added to 0.1 M PBS mixed with pluronic gel. After euthanasia, tissues were initially immersed for 4 hours at RT in a solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS (pH 7.4).

After fixation, tissues were then placed in a post fixative OsO4 1% solution diluted with NaPB buffer over night at 4 degrees C, tissue turned black showing positive reaction. Then the tissues were dehydrated in a graded series of ethanol and propylene oxide and embedded routinely in Spurr's resin. Ultra thin sections (70-90 nm) were stained with uranyl acetate and lead citrate and then viewed in a TEM. Cervical epithelial cell were subjectively analyzed for presence of paracellular spaces.

Statistical Analysis

Cervical tissue specimens were obtained from euthanized animals, which in some cases were previously ovariectomized. In all experiments involving two treatment groups, the students t-test was used. In Real Time PCR experiments, cervices of each treatment group were pooled and the difference in relative quantification between control and treated groups was determined using one way ANOVA.

Results

VEGF's Effects on Cervical Vascular Leakage

The present study shows that administration of VEGF at >40 ng could possibly induce infiltration of cervical tissue by serum proteins, as indicated by presence of EBD in the cervical tissue. Because EBD has a high affinity for serum proteins, presence of EBD in the tissue indicates leakiness of local tissue vessels. Successful uptake of EBD was confirmed visually as the animals gained a blue tint (Fig. 1). Animals administered with VEGF had an average increase of 0.0074 mg/ml EBD compared to a value of 0.0071 mg/ml of EBD in control group (Fig. 2) (p=0.775). The raw data did show a trend in that all animals' receiving VEGF treatment had a larger amount (mg) of EBD, than those in the control group.

VEGF's Effects on Cervical Edema

Having demonstrated that VEGF treatment could induce vascular permeability in cervical tissues of ovariectomized rats, we sought to investigate its effect on tissue edema. Consistent with our earlier vascular permeability studies, VEGF tended to induce a trend of increased edema in cervical tissues in a dose-dependent manner, i.e., 10 ng of VEGF induced an average increase of 0.009 g of tissue fluid compared to 0.016 and 0.024 g for 20 and 40 ng, respectively (data not shown) (p= 0.0573).

Thus, in subsequent studies of edema, we used the optimal dose (40 ng). In these studies,animals exhibited an average increase of 0.0273 g of tissue fluid compared to the control group, which had an increase of 0.0165 g (Fig. 3)(P=0.0961).

VEGF's Effects on Cervical TJ and CAM expression using Real-time PCR

Data generated from real-time PCR analysis showed the effects of VEGF on the expression profile of select TJ and CAM genes associated with intercellular junctions, specifically TJ and WBC tissue infiltration. The gene expression of TJ (caudin-1 and -2, occluding), and CAM (VCAM-1) were found to be elevated in VEGF-treated animals compared to control. These results were found to be consistent with those obtained from the vascular permeability, edema and cervical epithelial studies. VCAM-1, which is localized in the endothelial cells and strongly expressed during WBC extravasation had a relative fold increase of 4.482 compared to control group. Occludin showed a 3.209 fold increase compared to control group. Claudin-1 and -2 showed the most pronounced relative fold increases of 81.394 and 10.145, respectively, compared to control group (Fig. 4)(P= 0.0276).

VEGF's Effects on Cervical Epithelial Growth using SEM

SEM data analyzed from top and side-views revealed differences in the cervical epithelial structure between VEGF recombinant protein 164-treated animals, control and those treated with VEGF antagonist (Fig. 5). The most obvious was the dramatic increase in the degree of cervical epithelial folding. These findings are consistent with our earlier preliminary reports (Mowa et al., 2008).

In this study, we also investigated the structure of individual epithelial cells at a higher magnification (9,000x), and noted some subtle differences in the intercellular borders and shapes of cells between the different treatment groups (Fig. 5). Cells in VEGF-treated animals were less "fluffy" (microvilli) and had wider intercellular border or gaps compared to the control group. These data were consistent with that of TEM (below). Interestingly, in the VEGF antagonist-treated group, the cells had very poorly defined or irregular structures and borders, and had less abundant microvilli.

Histological Analysis of Cervical Epithelial Height

The discovery of VEGF-induced folds in the SEM studies prompted us to further investigate the effects of VEGF on cervical epithelial height using basic histology, SEM and molecular markers for proliferation (on-going study). Consistent with the earlier SEM findings, the height of cervical epithelia in VEGF-treated animals, based on our morphometric analysis, was greater than control by two-folds, i.e., 11.11 µm vs. 6.63 µm (Fig. 6) (P=5.659E-15).

VEGF's Effects on Cervical Epithelial Paracellular Space using TEM

Since VEGF induced vascular changes in cervical epithelial growth and TJ expression in the cervix, we sought to examine for presence of paracellular spaces between epithelial cells using TEM. Through this work we were able to visualize more of what we perceive to be paracellular spaces in VEGF-treated animals more than control group, based on our subjective observations (Fig. 7).

Discussion

The findings of the present study are that VEGF: (a) induces an increase in cervical epithelial sheet fold and cell height compared to control group; (b) causes a moderate increase in cervical tissue edema; (c) could induce increases in protein extravasation from vasculature, vascular permeability and paracellular spaces between epithelial cells, (d) alters the relative mRNA levels of TJ molecules (occludin, and claudin-1 and -2) and CAM (VCAM-1). Collectively, these findings suggest that VEGF plays a role in cervical tissue edema and epithelial growth, possibly via expression of TJ genes, vascular leakiness, protein extravasation and edema.

Cervical tissue dramatically increases during CR. Of the total cervical tissue growth, 75% is associated with the epithelia, and 55% with the stromal cells (Burger & Sherwood, 1998). The dramatic increase in cervical epithelial growth may reflect its role in CR. Indeed, recent reports by Mahendroo's group and others have suggested that cervical epithelial plays an important role in CR (Trends in Endocrinology, in print, 2010). Here, we show that VEGF has a dramatic effect on cervical epithelial folds and height in rats and mice using SEM and morphometrical analysis. These present results are consistent with our earlier preliminary data that showed an increase in epithelial folds most likely due to cellular proliferation as well as previous studies by others using different tissue types, such as fetal lung (Brown, England, Goss, Snyder & Acarregui, 2001). Further, there appears to be a temporal and spatial relationship between VEGF levels and its receptors with increase in

cervical epithelial cell mass during CR. The present study also shows that VEGF treatment affects cervical epithelial cell structure and microvilli density. Cervical epithelial cells in VEGF-treated animals, based on microscopic observations appeared to be more fluffy or swollen and had less defined boundaries than those in the control group, and microvilli were also less abundant. This is in contrast with VEGF blocker-treated animals where cellular borders were very poorly defined, were less organized, had an "unhealthy" appearance, as if degenerating, and showed a pronounced reduction in microvilli density. These data demonstrate that VEGF may not only influence the growth of cervical epithelial cells, but also their overall viability, structure, as well as microvilli density. To our knowledge, this is the first study demonstrating the effects of VEGF on cervical epithelial sheet. Taken together, these data show that VEGF likely plays an important role in CR, in part, by influencing growth of cervical epithelial cells, a cell type believed to play a central role in CR, according to recent studies. We currently are investigating VEGF's effects on molecular markers of epithelial proliferation in the cervix, including PCNA and BrdU. To date, the main factor reported to have an effect on cervical epithelial growth is relaxin. Relaxin increases proliferation of epithelial and stromal cells in the rat cervix during the second half of pregnancy (Burger & Sherwood, 1995). Interestingly, relaxin also stimulates expression of VEGF in cultured human endometrial cells (Unimori et al., 1999). It is possible that VEGF and relaxin in the cervix could have a synergistic relationship.

Cervical edema is one of the prominent features of CR in experimental animals and human (Burger & Sherwood, 1998; Myers, Paskalev, House & Socrate, 2008). A more recent report by House's group demonstrated significant increase in tissue hydration in pregnant vs. non-pregnant women, i.e., a difference of about 5% (Myers, Socrate, Tzeranis & House, 2009), which interestingly is comparable to our current data for VEGF-treated vs. control. Because of the temporal relationship between VEGF levels and cervical edema, and VEGF's edema-inducing effects in other tissue types, we sought to investigate its (VEGF) effects on cervical edema. The present study is the first to demonstrate VEGF's effect on cervical edema. It is important to state that because our dose response curve did not plateau, it is likely that increasing dosage (>40ng/animal) and sample size will widen the difference between treated and control groups. These studies are ongoing.

VEGF was first described as a potent vascular permeability factor (Senger et al., 1983). Compared to other endogenous potent vascular permeability factors, such as histamine, VEGF is 50,000 times stronger (Van Bruggen et al., 1999) and VEGF's role in tissue edema induction is well established, including in tissue types such as uterine, brain and nasal tissues. For instance, antagonism of VEGF action in the brain of stroke injury-induced edema significantly reduces swelling and hyper-permeability of brain microvasculature (Roberts & Palade, 1995; Van Bruggen et al., 1999, Dafni, Lansman, Schechter, Kohen & Neeman, 2002; Wang et al., 2001). In nasal polyps, expression of VEGF and KDR are localized in plasma cells and appear to be involved in signaling transduction (Ito et al., 1995). Other conditions that may induce expression of VEGF in the brain, which in turn induces cerebrovascular permeability, include hypoxia and ischemic brain injuries (Van Bruggen et al., 1999).

One of the main goals of the present study was to determine the effects of VEGF on cervical tissue edema. The exact mechanisms that underlie VEGF-induced vascular permeability and subsequent edema are not clearly known. Some studies suggest that increase in permeability is caused by a breakdown or disorganization of the molecules that constitute TJs between endothelial cells (Antonetti et al., 1999, Timmons et al., 2007). This conclusion is consistent with the present real-time PCR data. However, it is important to state that the present study did not investigate the specific cell types expressing TJ molecules and that other cell types, such as cervical epithelial cells, are also known to express TJ genes. It is possible, based on our current TEM data showing paracellular spaces between epithelial cells, in VEGF-treated groups, that expression of TJ molecules is also up-regulated in this cell type and that these spaces may possibly be routes of water absorption from the cervical lumen into the tissue. Studies addressing these informational gaps are currently ongoing in our lab. Aquaporins (water channels) have also been implicated in the absorption of water into cervical tissue (Anderson, Brown, Mahendroo & Reese, 2006). However, it is certainly clear, based on our Evans Blue data, that some of these molecular expressions are localized at local vasculature, as evidenced by increased vascular leakage. Evans Blue dye binds to serum proteins with a very high affinity, thus making tracking of serum proteins possible as they extravasate from the vasculature into the surrounding tissues. The accumulation of serum proteins in cervical tissues likely leads to generation of interstitial fluid-colloid osmotic pressure (π_{IF}). Senger et al., (1993) found similar data using the Miles assay. In their study, VEGF induced a dramatic response when injected intradermally in the flanks of guinea pig based on EBD.

The exact role of edema in CR is not completely understood. One possible role that we suggest is that it may provide turger pressure, as is the case in plants, i.e., the fluid pressure provides mechanical support to the otherwise softening tissues, allowing the cervix to maintain its barrier properties, while at the same time remodeling and preparing itself for the final process of fetal passage or birth. Thus, VEGF likely plays two seemingly opposing roles, namely enhancing cervical ripening, while maintaining the cervical barrier properties via edema formation.

TJ genes have recently been implicated in formation of vascular permeability under normal, pathological or experimental conditions (Gonzalez-Mariscal et al., 2003). Endothelia lining the vasculature have TJs that regulate passage of molecules, ions and water through the paracellular space. This property is commonly known as the gate or fence function of the TJ. To date, two components of the TJ have been identified, namely occludin and claudin, the latter being a protein family consisting of over 20 members (Gonzalez-Mariscal et al., 2003). It's been shown that over expression of mutant forms of occludin in epithelial cells lead to changes in the gate and fence functions of the TJ (McCarthy et al., 1996). Furthermore, in some epithelial cell lines, highly phosphorylated occludin molecules are selectively concentrated at the TJ, whereas, non- or less phosphorylated molecules will localize in the cytoplasm (Andreeva, Krause, Muller, Blasig & Utepbergenov, 2001). Therefore, occludin phosphorylation may play opposing roles in distinct biological systems or alternatively, phosphorylation of different residues may have dissimilar consequences. Clearly, occludin is a constituent of TJ filaments and its abundance is related to the degree of sealing between epithelia and endothelia cells (Saitou et al., 2000). Of interest, and relevance to the present study, it has been shown under experimental conditions that VEGF induces

rapid phosphorylation of TJ proteins occludin and zona occluden-1(ZO-1) (Antonetti et al., 1999), and VEGF increases bovine monolayer endothelial cell permeability by affecting occludin expression and TJ assembly (Wang et al., 2001). However, it is important to point out that most recent studies show that occluding knockout mice display a well developed TJ (Saitou et al., 2000). These confounding results led Tsukita and coworkers to search for another integral component of the TJ. Peptide sequencing revealed two proteins that were subsequently named claudin-1 and -2 (Gonzalez-Mariscal et al., 2003). They proposed that ionic selectivity at the TJ, determined by the specific claudins that constitute the junction vary and that the tightness of a TJ can be attributed to the combinations and mixing of different species of claudin (Mitic & Van Itallie, 2001). This conclusion is supported by the work of Furuse et al., (2002) that showed that when claudin-2 was introduced into a high resistance MDCK-1 cells their TJs become leaky and were similar in functionality and morphology to those in low resistance MDCK-2 cells, which normally contains high levels of claudin-2. The crucial task of claudin in the gate function of TJs was further highlighted by studies using claudin-1deficient mice. The epidermal barrier in these knockout mice is severely affected and leads to dehydration, wrinkled skin and death within 1 day of birth. In these mice it was also noted that occludin positive and claudin-1 deficient skin layers allow the passage of paracellular tracers, suggesting that the combination of claudin-1 and occludin is needed for the establishment of an effective paracellular barrier (Furuse et al., 2002). Of relevance to the present study, the claudin are also regulated during pregnancy and have the same temporal and spatial relationship as VEGF (Mowa et al., 2004; Timmons et al., 2007). However, the work in the cervix was focused on non-endothelial cells. Studies using other tissue types have shown that these TJ molecules are also expressed in the vascular

endothelial cells, such as in retinal endothelial cells, which provide blood-brain/retinal barrier in the eyes. In that study it was shown that VEGF affects the regulation of paracellular permeability through rapid phosphorylation of TJ proteins. Occludin tissue expression has been shown to affect the barrier properties of that tissue (Yaccino, Chang, Hollis, Gardener, & Tarbel, 1997).   Increased occludin content is associated with increased water permeability, and thus suggests that occludin phosphorylation may initiate allosteric conformational changes allowing acute increases in permeability, and degradation of the TJ protein may then contribute to the long term increase in permeability (Antonetti et al., 1999).

In conclusion, the present study has shown that VEGF induces expression of TJ genes vascular leakage, tissue edema, morphological changes in the epithelial layer of the cervix, such as increase in number of folds, epithelial cell height and paracellular spaces. We propose that increase in tissue edema is, in part, induced by vascular leakage and serum protein extravasation. Occludin and claudin-1 and -2 may mediate the VEGF-induced opening of the inter-endothelial "gates" leading to increased vascular permeability. Collectively, these findings suggest that VEGF plays a critical role in formation of cervical edema and epithelial proliferation that are both evident during cervical remodeling.

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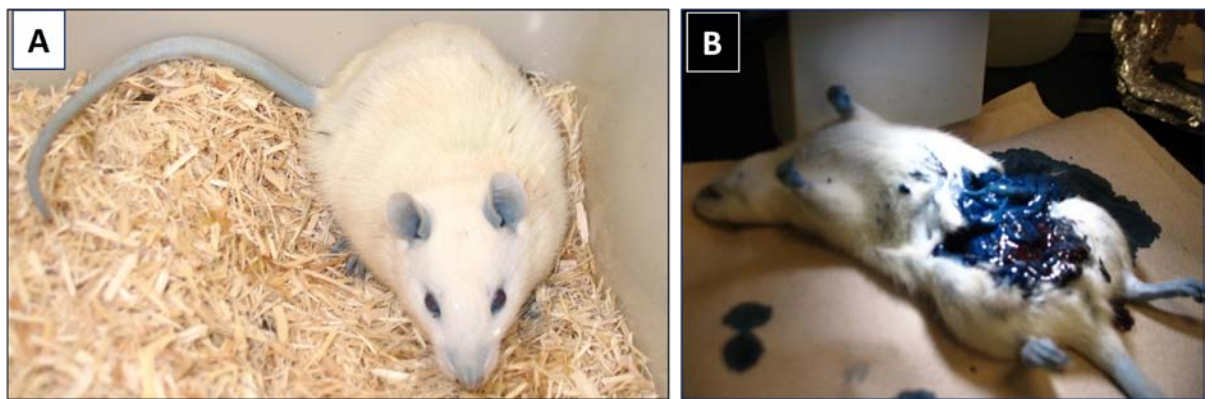
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Figure 1. *Determination of local blood vessel leakage using Evans Blue dye.* Shows successful uptake of Evans Blue dye administered IP (note "bluing"of animal in panel **A.** and **B**.) This dye has a high affinity for serum protein and is, therefore, used to track serum protein extravasation or determining local leakage of vessels, which leads to infiltration of local tissue by the dye.
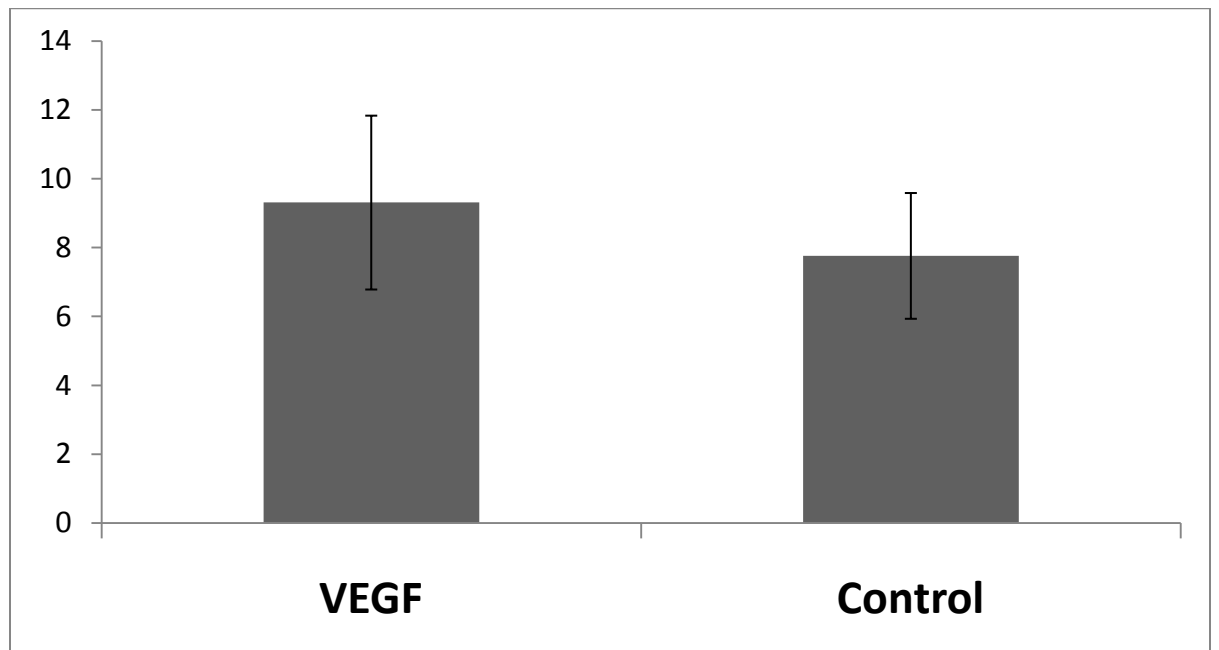
Figure 2. *Effects of VEGF on cervical vascular leakage*: VEGF recombinant protein 164 administered IP causes leaky vessels in the cervix of ovariectomized rats, as revealed by local tissue infiltration of Evans Blue dye. The concentrations of Evans Blue dye in the treated tissue increased (0.0074 mg/ml EBD) compared to control (0.0071 mg/ml EBD), as determined by spectrophotometry and standard curve (N=6, P= 0.775).
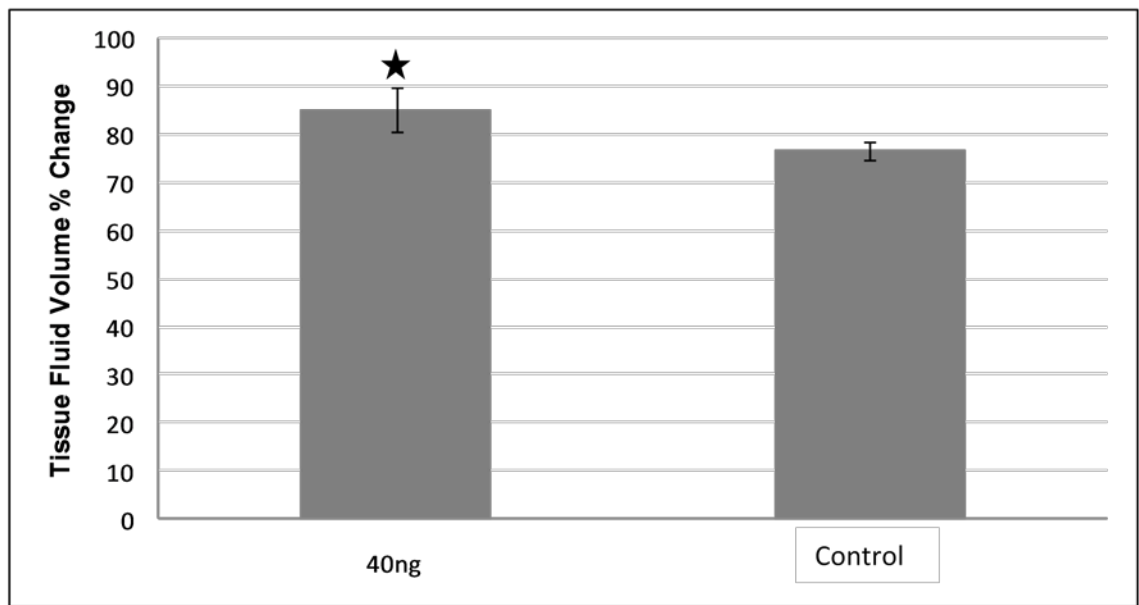
Figure 3. *Effects of VEGF on cervical edema***:** VEGF recombinant protein 164 administered IP causes leaky vessels in the cervix of ovariectomized rats leading to accumulation of fluid in the cervix (40ng/rat daily for 4 days) compared to control or vehicle only (n=8 , P=0.0961).
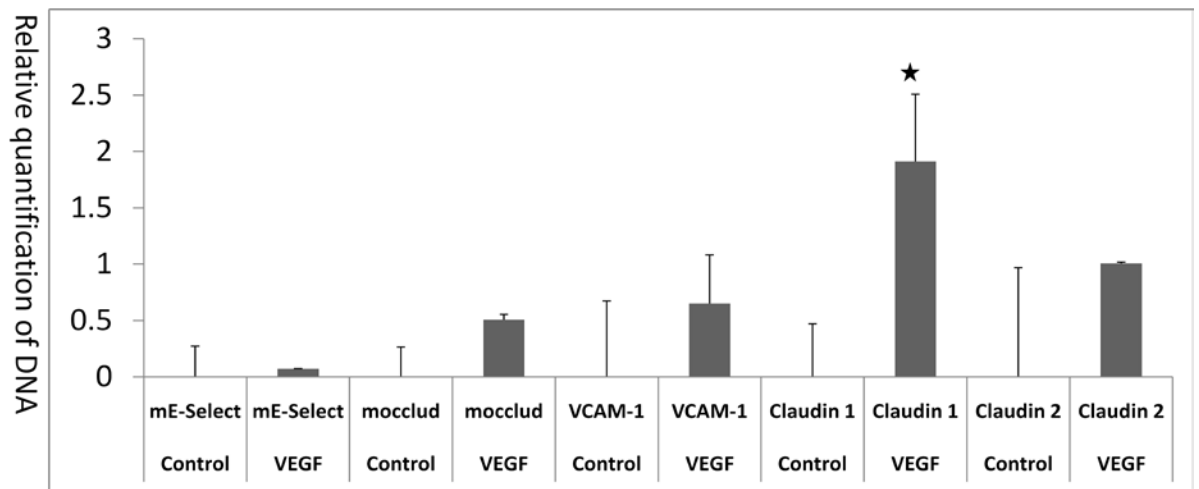
Figure 4. *Effects of VEGF on CAM and TJ gene expression, as revealed by real time PCR in non-pregnant ovariectomized mice*: Treatment type: Control and recombinant 164 VEGF protein. Cell adhesion molecules= VCAM-1, E-Selectin; Tight cell junction protein= Occludin and Claudins. All the genes show increase in mRNA levels as a result of VEGF treatment (n=5, P=0.0276).
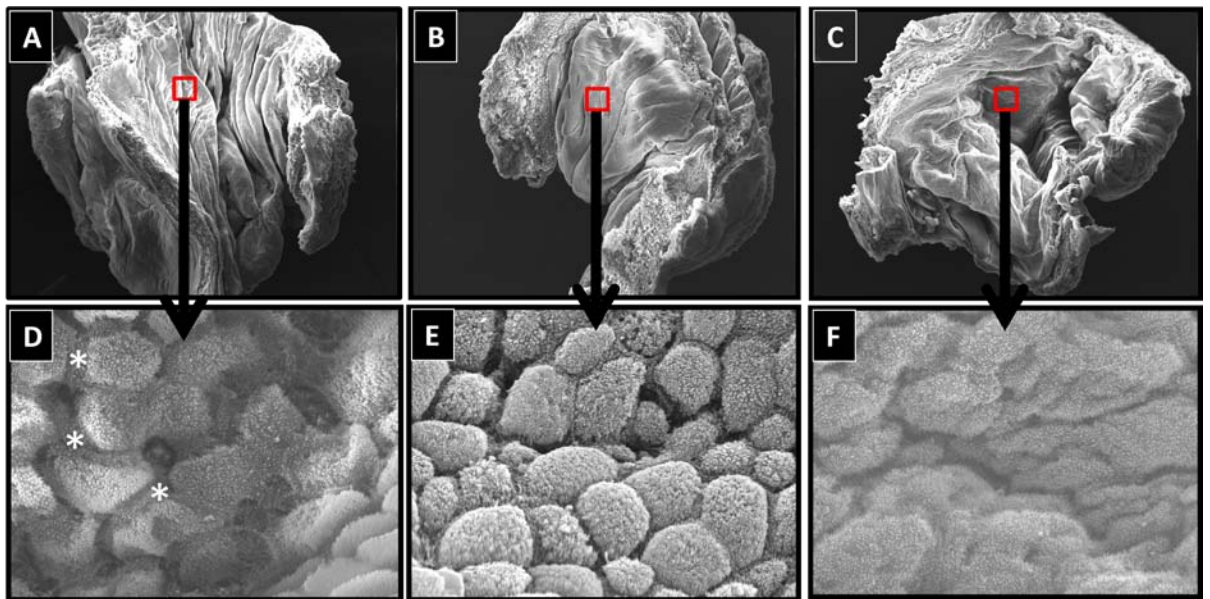
Figure 5. *Effects of VEGF on cervical epithelial folds:* **A., B.** VEGF recombinant protein induces epithelial folds of cervical tissue and inter-epithelial gaps (*), respectively, in ovariectomized mice compared to control **B.** treated with PBS only. panels. **C.** and **F.** show that blocking the action of VEGF via administration of its blocker reverses the effects of VEGF and "disfigures" cells (Low magnification, 149x) compared to control group .**E.** administered with PBS only (High magnification, 9,000x).
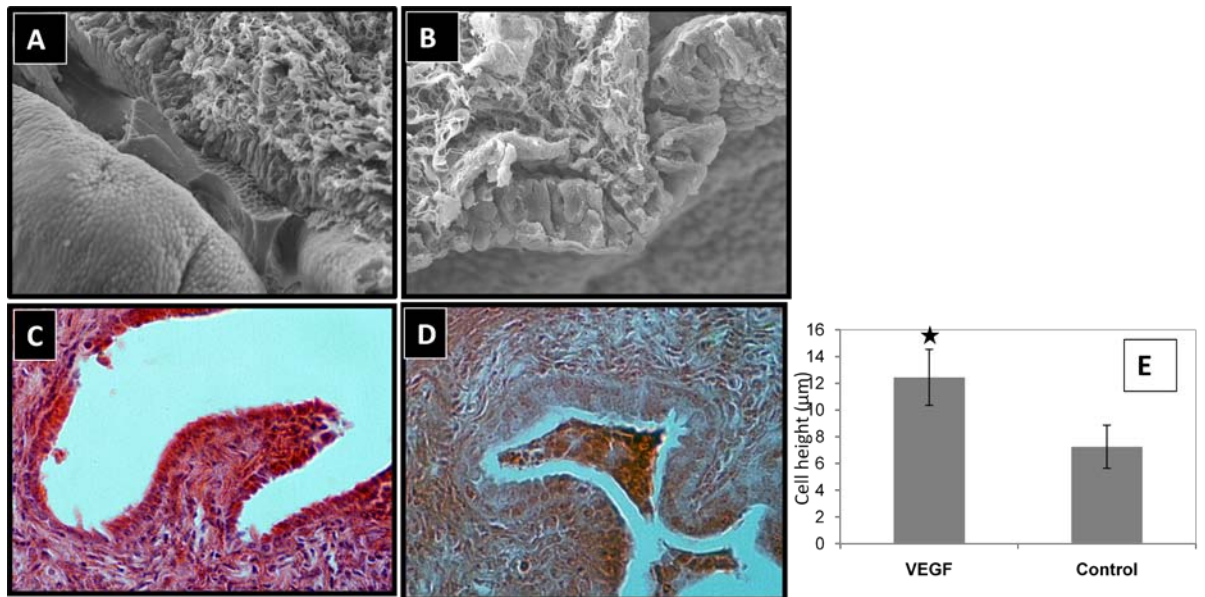
Figure 6. *Effects of VEGF treatment on cervical epithelial height.* Panel **A.** shows control (PBS) group of ovariectomized mice (1175x), which compared to VEGF-treated group (1862x) showed shorter cellular height, as revealed by SEM. Fig **C.** and **D.** shows basic histological sections of cervical epithelial tissues of control group **C.** and VEGF-treated group **D.** at 60x. The heights of these cells were measured and plotted as a graph. The average height of the cells in animals treated with protein were greater than control (N=20, P<0.05).
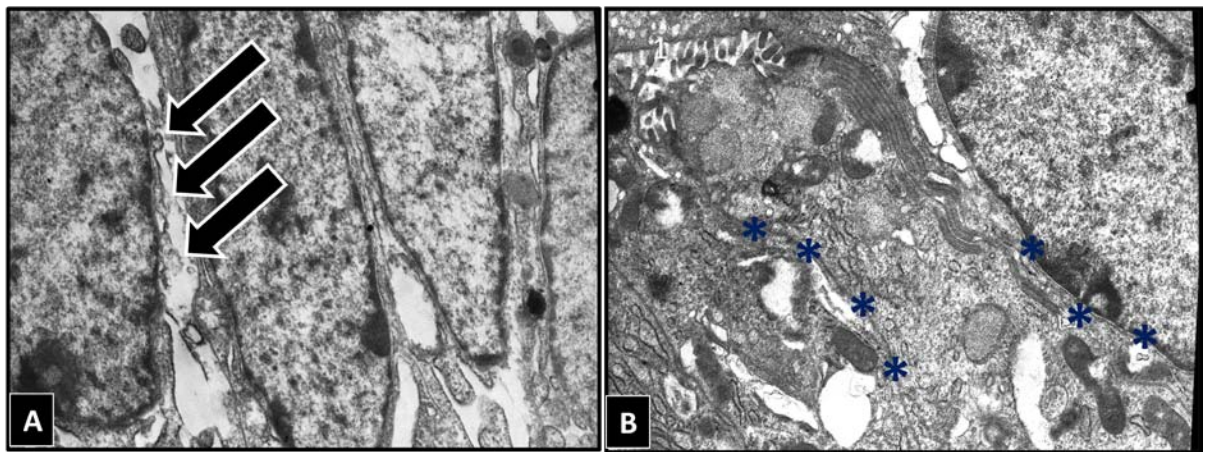
Figure 7. *Effect of VEGF on cervical epithelial paracellular space.* Administration of VEGF increases paracellular space (loosen tight junctions) in the epithelia of cervix **A**. Black arrows show paracellular spaces due probably to dissolution of the tight junctions connecting the cells (30,000x with TEM.) **B**. The paracelluar space or intercellular junctions of control mice show no dissolution, and are continuous between all cells (blue stars *) (30,000x.).

Biographical Sketch

Scott Brady Rhyne was born in Charlotte, North Carolina on February 18, 1984. He attended elementary Schools in Montevallo, Alabama, and in Catawba, North Carolina. He graduated from Bandys High School in 2002. The following fall he entered Appalachian State University in Boone, NC to study Biology with plans of attending veterinary school. In December of 2006 he was awarded The Bachelor of Science degree in Pre-professional Biology. In the fall of 2007 he accepted a master's candidate position at Appalachian State University, as well as a teaching assistantship in the Biology department. The Masters of Science was awarded in May 2010.

Mr. Rhyne was a member of the ASU Varsity Track and Field Team during his undergraduate career from 2002-2006 as a pole-vaulter. He has almost a decade of experience in the field of veterinary medicine, and at the time of this writing was an emergency veterinary technician at the animal emergency clinic of the high country. His Home address is 5305 Battle Run Drive, Catawba, North Carolina. His Parent are Mr. and Mrs. Tim and Cindy Rhyne.