Expression of Vascular Endothelial Growth Factor (VEGF) Receptors: Comparative immunohistochemical study of prepubertal, reproductive and postmenopausal endometria of female albino rats

Thesis
Submitted for Partial Fullfillment of the
M.Sc Degree in Histology

By
Asmaa Mohamed Elsayed
Demonstrator of Histology
Faculty of Medicine-Fayoum University

Under supervision of
Prof. Dr. Amal Mostafa Abbas
Professor of Histology
Faculty of Medicine-Cairo University

Dr. Mohamed Salah Elgendy
Assistant professor of Histology
Faculty of Medicine-Fayoum University

Dr. Noha Mohammed Afifi
Assistant professor of Histology
Faculty of Medicine-Cairo University

Faculty of Medicine
Cairo University
2010

ACKNOWLEDGMENT

At the beginning and first of all, I would like to present my obedience and all praise to Allah, who granted me the ability, support and all reasons of success to accomplish this work .

I hope to express my greatest appreciation and deepest gratitude to the praiseworthy, **Professor Dr. Amal Mostafa Abbas**, Professor of Histology**,** Faculty of Medicine, Cairo University, for keen supervision, guidance, support, encouragement and providing facilities and time throughout the work.

My greatest appreciation and deep thanks are to **Dr. Mohamed Salah Elgendy**, Assistant professor of Histology, Faculty of Medicine, Fayoum University, for his endless help as well as encouragement and kind support throughout my work.

All meanings of appreciation and gratitude to **Dr. Noha Mohammed Afifi,** Assistant professor of Histology, Faculty of Medicine, Cairo University, for her great efforts, continuous support and endless cooperation throughout this work .

I feel great honor to express my deepest gratitude and thankfulness feelings to my parents for their devotion, my husband for great efforts and for my brother for unlimited support.

Asmaa Mohamed Elsayed

Index

ABSTRACT AND KEY WORDS

Angiogenesis is an important process in endometrial development and embryo implantation, Vascular Endothelial Growth Factor (VEGF) regulates this process via interaction with its receptors; Flt-1and KDR. This work was carried out to study the immunoexpression of VEGF receptors in the endometrium at different ages and reproductive phases and to correlate them with the histological pictures in these phases.

Seventy female albino rats were included in this study. They were divided into three groups represent the prepubertal period, the reproductive period and the postmenopausal period, vaginal smears were done to determine the estrous cycle phases, the uteri removed from sacrificed rats and processed for staining with H&E and immunohistochemical staining for Flt-1&KDR.

The expression of VEGF receptors revealed to be highest in the pubertal age group with marked expression of these receptors in the proestrus phase followed by estrus phase and this supports the role of sex hormones especially estrogen hormone in regulating VEGF receptors expression. Flt-1 receptor has the predominance in the expression in endometrial epithelial, stromal and blastocyst cells while KDR receptor has the superiority in the endometrial endothelial cells. In conclusion the up-regulation of Flt-1 and KDR could be involved in regulating the endometrial endothelial cell proliferation and increasing the endometrial vascular permeability especially at the implantation sites.

KEY WORDS: Albino rats, Endometrium, Angiogensis, VEGF, Flt-1, KDR, Estrous cycle.

LIST OF ABBREVIATIONS

ANOVA: Analysis of variance.

DAB: Diaminobenzidine tetrachloride.

DNA: Deoxyribonucleic acid.

E2: Estrogen.

ECM: Extracellular matrix.

eNOS: endothelial nitric oxide synthase .

ER: Estrogen receptors.

FGF: Fibroblast growth factor.

FITC: Fluorescein isothiocyanate.

Flt-1: fms-like tyrosine kinase.

FSH: Follicle stimulating hormone.

HCG: Human chorionic gonadotropin.

HRP: Horse-Radish Peroxidase enzyme.

Hx&E: Hematoxylin and Eosin.

ICAM: Intercellular adhesion molecule.

IL: Interleukin.

KDR: Kinase insert domain containing region.

LH: Luteinizing hormone.

LIF: leukemia inhibitory factor.

M-MMP: Membrane bound matrix metalloproteinase

MMP: Matrix metalloproteinase.

mRNA: messenger ribonucleic acid

NK: Natural killer.

P4: Progesterone.

PBS: phosphate buffered saline.

PR: Progesterone receptor.

S.D: Standard deviation.

sVEGFR: soluble form of VEGFR.

TGF: Transforming growth factor.

uNK: uterine natural killer.

VEGF: Vascular endothelial growth factors.

VEGFR: Vascular endothelial growth factor receptor.

VRF: VEGF-related factor.

LIST OF TABLES

Introduction and Aim of Work

Angiogensis, the sprouting of new vessels from the existing vasculature, is fundamental for endometrial development and differentiation necessary for implantation and the uterine changes associated with pregnancy. In the absence of implantation, angiogenesis is also required to support endometrial regeneration after shedding of the uterine surface and to support the proliferation of the endometrium during the reproductive cycle under the control of estrogen and progesterone *(Perrot-Applanat, 2000).* These extremely rapid growth and regression changes in the endometrium should be accompanied by equally rapid changes in the rates of blood flow *(Halder et al., 2000).*

Vascular endothelial growth factor (VEGF) is a proangiogenic growth factor that acts as a potent mitogen for vascular endothelial cells *(Reynolds et al., 2000)* and has been shown to act as a cytoprotective agent, protecting these cells from apoptosis *(Tran et al., 2002).*

VEGF exerts its cellular effects through interaction with its transmembrane tyrosine kinase receptors Flt-1 (VEGF-R1) and KDR (VEGF-R2) which bind the ligand with high affinity *(Ferrara and Gerber, 2001).*

KDR is the principal mediator of the angiogenic effect of VEGF and its importance is highlightened by failure of KDR-null mice to develop organized blood vessels resulting in lethality between embryonic days 8.9 and 9.5 *(Shalaby et al., 1995).*

The role of Flt-1 in angiogenesis is less apparent and is a subject of debate. Some studies postulated that, during early embryogenesis, Flt-1 is important in vascular modeling and Flt-1-null mutants die at med-gestation with vascular overgrowth and disorganization *(Fong et al., 1995).*

Some reports have indicated that Flt-1 has limited signaling activity and may act as a decoy receptor *(Matsumolo et al., 2002),* whereas other studies have implicated Flt-1 in mediation of endothelial proliferation *(Zeng et al., 2001),* chemotaxis *(Yang et al., 2002)* and cell survival *(Le Couter et al., 2003).*

Reports were conflicting as regards the role of female sex hormones; estrogen and progesterone, in controlling angiogenesis. There is also an extreme importance for angiogenesis in early pregnancy to confirm successful implantation of mammalian embryos, at the blastocyst stages, into the uterine endometrium *(Perrot-Applanat, 2000).*

Aim of work:

In the present study female albino rats were used as animal models to study the intensity of immunostaining for VEGF receptors (Flt-1 & KDR) in the endometrium at different ages including the prepubertal and postmenopausal ages, which are known to have negligible levels of sex hormones, as well as the pubertal age with varying levels of these hormones according to the reproductive phase. Also immunoexpression of the two receptors were studied in the endometrium during early pregnancy. This could help in proper understanding of the vascular remodeling and angiogenesis of the endometrium.

The Histology of Endometrium

The endometrium is the descriptive term for the lining mucosa of the uterine cavity. It is primarily specialized to allow embryo implantation. It also acts as a secretory mucosal barrier, providing continuous protection against pathogens that gain access to the uterine cavity *(Simon et al., 2002).*

Endometrium is also a dynamic tissue that; in response to ovarian estrogen and progesterone exposure; undergoes well characterized cyclic proliferation, differentiation and tissue breakdown. In humans, these cyclic phases occur on a monthly basis called the menstrual cycle *(Jabbour et al., 2006 and Gargett, 2007).*

The endometrium consists of an epithelium which is a mixture of ciliated and secretory simple columnar cells. Simple tubular glands extend from this epithelium and sometimes branch in their deeper portions, near the myometrium. The epithelium of the uterine glands is similar to the superficial epithelium, but ciliated cells are rare within the glands *(Critchley et al., 2001b).*

The underlying lamina propria, commonly called the endometrial stroma, is formed of loose connective tissue rich in fibroblasts and contains abundant ground substance. Connective tissue fibers are mostly made of collagen type III *(Junqueira & Carneiro, 2005).*

Endometrial stromal cells include T and B lymphocytes, uterine natural killer (uNK) cells which have a unique phenotype distinguishing them from peripheral blood NK cells *(King, 2000 and Henderson et al., 2003).* Neutrophils are also apparent among the stromal cells. They synthesize and release a wide range of immunoregulatory cytokines and thereby initiate and augment cellular and humoral immune responses. Neutrophils also express antiproteinase and antimicrobial molecular elafin *(King et al., 2003).*

Other constituents of the endometrial stroma are macrophages which express matrix metalloproteinase-9 (MMP-9) and membrane bound matrix metalloproteinase-1 (M1-MMP). Both these enzymes are involved in the breakdown of extracellular matrix and have been proposed to play a role in menstruation *(Jeziorska et al., 1996 and Zhang et al., 2000).* Furthermore, macrophages are also a source of vascular endothelial growth factor, VECF *(Smith, 1998).*

Mast cells are also present in the endometrial stroma expressing mast cell tryptase and matrix metalloproteinase -1 (MMP-1). They could have a role in upregulation of matrix metalloproteinase (MMP) before the onset of menstruation *(Jeziorska et al., 1995; Zhang et al., 1998 and Milne et al., 2001).*

The endometrial layer can be subdivided into two zones; the basalis is the deepest one, adjacent to myometrium; it contains lamina propria and the closed tips of uterine glands. The second zone is the functionalis; it contains the remainder of the lamina propria and the glands, as well as the surface epithelium. Whereas the functionalis undergoes profound changes during the menstrual cycle, the basalis remains mostly unchanged *(Padykula, 1991; Brenner et al., 2003 and Slayden & Brenner, 2004).*

Gynecological pathologists define four zones of endometrium at successive levels from the free surface to its base. The functionalis is divided into zone I consisting of the surface epithelium and the adjacent stroma, and zone II which includes the straight upper segments of the glands. The basalis is devided into zone III containing the branching lower segments of the glands, and zone IV, the bottoms of the glands and their associated stroma. This zonation is useful in examination of endometrial biopsies for accurate establishment of the stages of the menstrual cycle *(Bloom & Fawcette, 1996).*

Architecture of the endometrial vasculature

Blood supply to the endometrium arrives through the radial arteries, which arise from the arcuate arteries within the myometrium. After passing through the myometrial–endometrial junction, the radial arteries split to form the smaller basal arteries that supply the basal portion of the endometrium and the spiral arterioles which continue up towards the endometrial surface *(Ramsey, 1982).*

The spiral arterioles, which supply the functional layer of the endometrium, have a distinctive coiled appearance that becomes more pronounced during the secretory phase of the menstrual cycle. Each spiral arteriole provides blood to a uterine luminal surface area. Capillaries branch from the spiral arterioles at all levels supplying the stroma and periglandular networks. Just below the endometrial surface the spiral arterioles break up into the prominent subepithelial capillary plexus. This plexus drains into a number of venules that pass down through the endometrium, collecting blood from other capillaries on the way. The numerous venules join to form veins which pass out of the endometrium into the myometrium. The basal portion of the endometrium and its vasculature remains relatively unchanged throughout the menstrual cycle. By contrast, the functional layer of the endometrium is constantly changing in response to circulating sex steroids *(Ferenczy, 1987).*

The strength of small arterial blood vessels in the endometrium is derived from combination of endothelial cells, basement membrane, and cells with smooth muscle character that surround this membrane and resemble the decidual cells of pregnancy. These perivascular cells also express inflammatory agents such as prostaglandins and cytokines *(Milne et al., 1999 and Aplin, 2002).*

The basement membrane of the small arterial blood vessels in the endometrium is comprised of collagen type 4, fibronectin, and glycosaminoglycans and varies from 50 to 350 nm in thickness, with an increase occurring during the luteal phase of the cycle. Certain components such as heparan sulfate proteoglycan show a decrease in their level the menstrual phase of the cycle *(Kelly et al., 1995)* which may reflect destabilization of the vessels. The basement membrane can be broken down by various matrix metalloproteinases (MMPs) and it is the control of these MMPs by steroid hormones that is one conduit for progesterone action. The matrix components of the basement membrane are synthesized by neighboring (perivascular) stromal cells under the influence of progesterone. In particular, Progesterone stimulates synthesis of fibronectin *(Zhu et al., 1992)* and thrombospondin *(Iruela-Arispe et al., 1996).*

The Menstrual Cycle

In primates, as humans, the endometrium goes through a continuous sequence of histological cyclic changes; the menstrual cycle; which is divided into three phases:

Proliferative (Follicular) Phase:

Beginning at the end of menstrual flow and continuing for 12-14 days and is overall regulated by estrogen originating from the developing ovarian follicle, there is a three to four fold increase in the thickness of the endometrium. The initial stage of endometrial repair involves migration of epithelial cells from protruding stumps of basal glands over denuded surface. Many cells are found in mitosis, both in epithelium and in the stroma, as the surface epithelium is being restored and the tubular glands are increasing in length. The spiral arteries, shortened in the sloughing of the functionalis, are growing. As the glands lengthen, they become sinuous and their columnar epithelium begins to accumulate glycogen. During the proliferative phase of the cycle, the endometrium has increased from a postmenstrual thickness of 0.5mm to 2-3mm *(Okulicz & Scarrell, 1998 and Chan et al., 2004).*

Secretory (luteal) Phase:

The secretory phase starts after ovulation and under the effect of progesterone secreted by the corpus luteum. The epithelial cells continue accumulating glycogen below their nuclei *(Slayden & Brenner, 2004 and Spencer et al., 2005).*

Later, the amount of glycogen diminishes, and glycoprotein secretory products dilate the lumens of the glands. One important feature of this phase is that the glands become highly coiled and in this phase, the endometrium,

reaches its maximum thickness (5mm) as a result of the accumulation of secretions and of the edema in the stroma *(Junqueira & Carneiro, 2005).*

In late secretory phase uterine natural killer cells (uNK), which are very few in the proliferative phase, constitute the majority of connective tissue cells population. They are seen in close contact to endometrial glands and blood vessels. In absence of pregnancy, uNK may be important in the initiation of menstruation *(King, 2000 and Henderson et al., 2003).*

Menstrual (Destructive) phase:

When fertilization of the oocyte and implantation does not occur and the corpus luteum ceases functioning, the consequent rapid decrease of blood levels of progesterone and estrogens causes menstruation. Several factors are involved in the shedding of the endometrium, such as cycles of contraction and relaxation of the spiral arteries, activation (by lack of progesterone) of locally produced matrix metalloproteinase, and local release of prostaglandins, cytokines, and nitric oxide. These factors lead to breakdown of blood vessel walls and basement membranes as well as collagen of the endometrial lamina propria and differentiated or decidualized stromal cells undergo apoptosis. Blood vessels rupture above the constriction, and bleeding begins, consequently, part of the functional layer of the endometrium becomes detached *(Salamonsen, 2003 and Jabbour et al., 2006).*

Some components of the basement membrane of blood vessels such as heparan sulfate proteoglycan decrease during the menstrual phase of the cycle, Because of the overall negative charge on many components of the extracellular matrix, these molecules can act as tethering points for growth factors. Thus, loss of molecules such as heparan sulfate proteoglycan during the menstrual process may lead to an increase in growth factor availability that will induce the re-growth of endometrial tissue *(Kelly et al., 1995).*