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A Critical Review of Endometriosis Pathology

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A Critical Review of Endometriosis Pathology

by

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Abstract

Endometriosis is a complex, multifactorial, reproductive disorder present in approximately 10-15% of adult women between the ages of 25-35. This disorder occurs when endometrial glands and stroma grow ectopically on the surface of the ovaries, pelvic peritoneum, fallopian tubes, and the uterus. Endometriosis causes varying degrees of painful symptoms and infertility in infected individuals. Three main theories of endometrial accumulation attempt to explain the etiology of this elusive disease. There have been various approaches to staging of endometriosis symptoms that attempt to standardize classification as well as predict pain and infertility. Angiogenesis, necessary to the survival of endometrial tissue, along with immune dysfunction and evasion have been examined as possible contributing factors to the development of endometriosis. Certain angiogenic factors are upregulated in ectopic endometrial tissue and endometriotic lesions, while decreased cytotoxicity of T cells is shown to be an abnormal immune process observed in individuals with this disorder. Other factors including exposure to environmental toxicants, diet, and population specific polymorphisms have also been examined for their role in this disease. While various genetic variations have been identified as increased risk factors for endometriosis in certain populations, there has not been a specific genotype that demonstrates increased risk for this disease in all populations. Endometriosis-induced animal models, which include rodents and non-human

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primates, are primarily responsible for the advancement of our understanding of this disease. Although limitations exist for animal models, they have been an important contributor to current research. There is no cure for endometriosis, but various treatment options exist for both pain and infertility. The purpose of this thesis is to review current literature relevant to the etiology, development, and treatment of endometriosis.

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List of Abbreviations

AFS .... American Fertility Society

AhR ....Aryl Hydrocarbon Receptor

APC .... Antigen Presenting Cell

ARNT .... Aryl Hydrocarbon Nuclear Translocator Protein

ASRM ....American Society for Reproductive Medicine

COC...Combination Oral Contraceptives

COX...Cyclooxygenase

CTL..Cytotoxic T Cell

DC.. Dendritic Cell

DIE...Deep Infiltrating Endometriosis

E1 ....Estrone

E2 ....17β-Estradiol

E3 ....Estriol

EE..Ethinyl estradiol

EFI...Endometriosis Fertility Index

FasL ....Fas Ligand

FGF ....Fibroblast Growth Factor

FSFI.. Female Sexual Function Index

FSH...Follicle Stimulating Hormone

GnRH ....Gonadotropin-Releasing Hormone

HSC ....Hematopoetic Stem Cells

IFN.. Interferon

IL..Interleukin

ISO ....(S, R) 3-(4-hydoxyphenyl)-4,5-dihydro-5-isoxazole acetic methyl ester

IUD ....Intrauterine Device

IVF ....In-Vitro Fertilization

LF .... Least Function

Chapter I: Introduction

Endometriosis is a female reproductive disorder present in approximately 15% of adult women between the ages of 25-35 (Macer 2012). This disorder occurs when the endometrial tissue (cells that line the uterus) grows in other areas of the body. This abnormal growth of endometrial tissue, is referred to as ectopic endometrium and can occur anywhere in the body, but is most commonly observed in the pelvis—on the outer surface of the ovaries, fallopian tubes, or the uterus.

The cyclic shedding of ectopic endometrium within the abdomen can cause irritation; lower back, intestinal, or pelvic pain, heavy menstrual periods or spotting between periods, dysmenorrhea, dyspareunia, and infertility (Constanti 1998). The etiology of endometriosis is unknown, however, there are three major theories that attempt to explain the origin of this disease. The most commonly accepted theory is that of retrograde menstruation proposed by John A. Sampson, M.D. in 1927 described below (Alford 2010). Meyer's theory promotes the idea of coelomic metaplasia and Halban's theory focuses on endometrial spreading via the vasculature and lymphatics.

Endometriosis is an estrogen dependent disease. When estrogen levels increase during the menstrual cycle the ectopic tissue grows and then regresses in the absence of estrogen similar to the activity of normal uterine endometrium (Constanti 1998). Current research suggests that genetic variation and

polymorphisms contribute to the disease within certain populations. A few animal models have been developed to study endometriosis using mice, rats, and nonhuman primates. While differences between the rodents' estrous cycle and a woman's menstrual cycle prevent a direct comparison of disease findings, there is still much to learn about disease etiology using these models. A specific species of baboon has also been developed to simulate endometriosis and has a similar menstrual cycle to that of a human, but it is difficult to perform extensive experimental manipulations in this model due to the high cost associated with utilizing these animals. There are specific advantages and disadvantages to the study of each endometriosis animal model which will be discussed below.

Current research also explores other factors potentially influencing the development of endometriosis. These factors include angiogenesis and immune response. Angiogenesis is the formation and growth of new blood vessels. This is required for the development and survival of endometrial tissue; without a blood supply there would be no oxygen or nourishment necessary for maintaining this tissue. Studies have explored the role of angiogenic factors in endometriosis development.

Another such factor includes dysfunction of the immunological response to the misplaced endometrium. The dysfunction of necessary immune cells normally recruited to the uterine lumen during the menstrual cycle could play a role in the ability of ectopic endometrial tissue to implant and proliferate. Immune cells such as macrophages and lymphocytes required to clear the normal shedded endometrium could lead to pathology if unable to perform their normal function. Also, environmental factors, such as exposure to environmental

toxicants and chemical pollutants, and certain foods such as fatty acids have been studied for their potential role in the development of this disease.

   While there is no cure for endometriosis there are treatments that help reduce the symptoms (Senapati 2011). This review will discuss the current research and literature as it pertains to the possible origins, development, genetic variation, pathology, and treatment of endometriosis.

Chapter II: The Menstrual Cycle

2.1 Overview

Endometriosis is defined as glandular and stromal tissue of the endometrium outside of the uterus (Burney 2012). Ectopic endometrium, similar to eutopic endometrium, proliferates and sheds in response to hormonal fluctuations. These fluctuations are due to physiological changes in the ovary and the regulation of these changes by the hypothalamic-pituitary axis. The normal changes that characterize the phases of the menstrual cycle are responsible for variations in the histology of the reproductive tract, including the endometrium, the understanding of which will provide the basis of endometriosis pathogenesis. The changes that occur during the menstrual cycle in response to hormonal regulation, changes in the histology of the reproductive tract, and functional and mechanistic differences between eutopic and ectopic endometrium will be reviewed in this chapter.

2.2 Phases of the Menstrual Cycle

Menarche, the initiation of menses and fertility, occurs in young women between the ages of 9-14 (Ross 2011). During this time there are changes that occur to a woman's reproductive organs. The ovaries produce the steroid hormones, progesterone and estrogen, which along with neural activity regulate these changes. The ovarian cycle in women consists of two phases: an estrogen

dependent follicular phase and a progesterone dependent luteal phase. Due to the variation in hormones that are produced during the menstrual cycle, changes occur within the uterus that promote an environment for embryo implantation.

The hypothalamic-hypophyseal portal system is ultimately responsible for regulating the changes in plasma hormone concentration (Constanti 1998). The hypothalamic-hypophyseal portal system moves blood between a primary capillary bed in the median eminence and a second capillary bed in the anterior pituitary. When hypothalamic peptide hormones are released into the portal system they are transported by the portal veins directly to the anterior lobe.

Through the actions of Gonadotropin-releasing hormone (GnRH), the hypothalamus controls secretion of the gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary gland (Constanti 1998). GnRH is released in a pulsatile fashion from the hypothalamus into the portal system and travels to the anterior pituitary, where it acts on its receptors to cause release of the gonadotropin hormones. During the follicular phase of the menstrual cycle the plasma levels of FSH and LH slowly rise; this causes the ovarian follicles to mature. As estrogen levels increase in circulation, it negatively feeds back on both the anterior pituitary gland and the hypothalamus, this decreases the amount of estrogen produced by the ovarian follicular somatic cells.

The initial rise in estrogen characterizes the follicular phase occurring the first 14 days of the menstrual cycle (Ross 2011). This phase is also referred to as the proliferative phase, because there is proliferation of granulosa and theca cells of the ovarian follicle and of the endometrium. Both FSH and estrogen also increase the sensitivity of granulosa cells for FSH by increasing the number of

surface receptors for this hormone. By the late follicular phase there is a switch as high estrogen begins to work in a positive feedback mechanism at the level of the anterior pituitary and hypothalamus. This positive feedback mechanism is responsible for the LH surge and increased levels of progesterone that occur during the luteal phase. Increased estrogen levels are due to a proliferation of estrogen secreting follicular cells and through estrogen action on both the hypothalamus and anterior pituitary. High estrogen levels due to positive feedback and the surge of LH and FSH cause release of the secondary oocyte and surrounding cells from the ovary.

The luteal phase, days 15-28 of the menstrual cycle, is dependent on high progesterone levels (Ross 2011). Progesterone is produced by the residual follicular cells, which form the corpus luteum. The progesterone prepares the endometrium for implantation by causing the endometrium to thicken, vascularization to increase, and proliferation to decrease. If the secondary oocyte is not fertilized in the fallopian tubes, it passes through to the uterus. In the absence of fertilization and implantation into the uterine wall, the corpus luteum regresses, resulting in a decline in estrogen and progesterone levels. Without the sex hormones to maintain the endometrium degradation occurs (Gray 2005). The shedding of the endometrium with the exception of the basal layer typically lasts about four days and its onset starts the next cycle.

2.3 Role of Estrogen in the Menstrual Cycle

The steroid hormones, estrogen and progesterone, are produced in the ovaries by a process called steroiodogenesis (Ross 2011). These hormones, formed from a cholesterol precursor, are synthesized in a cyclic manner and are

responsible for the changes that occur in the female reproductive system. Estrogens are critical regulators of normal reproductive function (Heldring 2007). Three forms of estrogen exist as shown in Figure 2.1: 17β-estradiol (E2), estriol (E3), and estrone (E1). E2 is the predominant and most potent form of estrogen in adult pre-menopausal women. E3 increases during pregnancy and E1 is the major form of estrogen in post-menopausal women.

Estrogen is important in the development of both internal and external sex organs and is also responsible for the female sex characteristics that develop in young women at puberty. Two types of estrogen receptors exist in the body and establish the physiological functions of estrogen, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Burns 2012). Both receptors are part of the nuclear receptor family of transcription factors and are vital to the function of the reproductive tract, breast, bone, and brain (Heldring 2007). Accepted research shows marked functional differences between these two receptors, including the opposite effects these receptors induce on promoters of genes involved in proliferation. Estrogen bound to ERα has been shown to induce the transcription of pro-proliferative target genes, such as cyclin A and c-Myc mRNA (Zhang 2009), whereas ERβ is responsible for activating anti-proliferative target genes and producing anti-inflammatory effects (Heldring 2007).

As previously mentioned, LH is released from the anterior pituitary and stimulates theca cells to produce testosterone. Under the actions of LH, the lipid-soluble molecule cholesterol is converted to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme complex in theca cells (Constanti 1998). Through a series of reactions pregnenolone is converted into testosterone,

which traverses the theca cell and basement membrane and diffuses into granulosa cells. FSH binding to its receptor on granulosa cells increases aromatase enzyme synthesis and activity, which is responsible for converting testosterone into estradiol.

Progesterone, secreted by the corpus luteum during the luteal phase of menses, enables the uterus to adapt for pregnancy by allowing for changes in the endometrium (Ross 2011). The rise and fall of estrogen and progesterone characterize the menstrual cycle. During the menstrual phase of the uterine cycle, regression of the corpus luteum occurs, and decreases in estradiol and progesterone allow for the endometrial lining to shed. This cyclic sloughing and regenerating feature distinguishes endometrial tissue and thus endometriosis from other pathology such as cancerous growths.

2.4 Histology of the Female Reproductive System and Endometrium

The female reproductive tract is composed of a pair of almond-shaped ovaries and fallopian tubes that come together at the uterus (Ross 2011). The primary functions of the ovaries are oogenesis and steroidogenesis. A center medullary region and an outer cortex form the bulk of the ovarian structure, while the surface is covered in a layer of simple cuboidal cells, referred to as the germinal epithelium. The medulla contains loose connective tissue, blood vessels, and lymphatics; while the cortex surrounding the medulla contains the follicles surrounded by smooth muscle fibers. The female germ cells, oogonia, undergo mitosis and stop in prophase I of meiosis before they become primordial follicles (Blake 2012). This occurs in the fetus. The long period of meiotic arrest leaves the primary oocyte vulnerable to potentially harmful

environmental influences that could develop years after birth. Simple squamous follicular cells surround the primary oocyte forming the primordial follicle.

At the start of the menstrual cycle, due to increasing levels of FSH, a group of primordial follicles enlarge and become primary follicles (Constanti 1998). The primary and antral stages of development are referred to as the growing follicle (Blake 2012). The process of developing an ovulatory follicle from this early stage takes 3-4 menstrual cycles. The transition between primordial follicle and primary follicle involves morphological changes; the oocyte begins to enlarge and the surrounding squamous cells become cuboidal (Ross 2011). The change from squamous to cuboidal of the surrounding follicular cells characterizes a primary follicle. The growing oocyte emits a gel-like, nutritive protein material called the zona pellucida, which separates it from the follicular somatic cells. The single layer of follicular cells undergoes rapid proliferation and forms the granulosa cell layer. The stromal cells surrounding the follicle form a sheath of connective tissue called the theca folliculi made up of a theca interna and theca externa. The theca interna is highly vascularized and contains LH receptors, while the theca externa contains smooth muscle.

As the primary follicle continues to grow into the antral follicle the granulosa cell proliferates and becomes 6-12 cell layers thick (Ross 2011). The granulosa cells that are associated with the oocyte are referred to as the cumulus oophorus and the antrum is formed from the combined fluid-filled cavities between granulosa cells. Through a process not completely understood one follicle continues to grow and becomes the dominant or Graafian follicle extending throughout the entire cortex of the ovary and causing a bulge. The other follicles at different stages regress and are reabsorbed through atresia.

The LH surge causes ovulation in which the secondary oocyte and surrounding cells are released from the Graafian follicle (Ross 2011). The cells of the cumulus oophorus immediately surrounding the oocyte and remain with it at ovulation are called the corona radiata. The secondary oocyte and the corona radiata exit the ovary through a break in the germinal epithelial layer and move into the fallopian tubes. The secondary oocyte is a product of the first meiotic division. The daughter cells of the primary oocyte each receive the same amount of chromatin, however, one daughter cell receives a majority of the cytoplasm and becomes the secondary oocyte. The second daughter cell with a lesser amount of cytoplasm becomes the first polar body. After ovulation the secondary oocyte is only viable for about 24 hours, if fertilization fails to occur, the secondary oocyte degenerates.

The wall of the fallopian tubes, also called uterine tubes or oviducts, are made up of three layers (Ross 2011). The outer most layer is the serosa or peritoneum, the middle layer the muscularis, and the innermost layer the mucosa. The mucosal lining of the uterine tubes contains both ciliated and non-ciliated simple columnar epithelium. The cilia, composed of an internal core of microtubules, are apical modifications that exist on the surface of epithelial cells. These microscopic structures beat together in order to aid in moving the secondary oocyte or embryo (if fertilized) from the oviducts into the uterus. The randomly dispersed non-ciliated peg cells provide nourishment for the embryo.

The uterus is a muscular organ about 7 cm long, 4 cm wide, and 2.5 cm thick (Ross 2011). It is divided into three regions: the body, the fundus, and the cervix. The uterine wall of the body and the fundus is composed of the endometrium and myometrium. The myometrium is the smooth muscle layer of

the uterus that has the ability to contract in response to stimulation (Blake 2012). Oxytocin stimulates uterine contraction at the end of pregnancy by acting on its receptor and through initiation of prostaglandin synthesis. Oxytocin has an increased effect due to estrogen, which sensitizes the myometrium to oxytocin by up-regulating its receptor. Progesterone has the opposite affect and causes suppression of uterine contractions.

The mucosal lining of the uterus is called the endometrium, which can be divided into the stratum basale and the stratum functionalis (Ross 2011). The stratum basale, or the basilar zone, gives rise to the stratum functionalis that grows and is then shed each month. The monthly changes that occur in the endometrium correspond to the hormonal changes of the follicular and luteal phases of the ovarian cycle (Gray 2005).

The endometrial tissue that lines the uterine cavity is made up of simple columnar epithelium (Young 2000). The supporting stroma contains simple tubular glands. The endometrium is 1-2 mm thick at the beginning of a cycle and the glands are straight, non-secreting, and lined with columnar epithelium (Gray 2005). Early in the proliferative phase, re-epithelialization or resurfacing occurs, in which the remnant basilar zone of the uterine glands proliferate and migrate to cover the endometrial surface stripped from its mucosa by menstrual shedding. This process concludes about 5-6 days after the start of shedding.

As ovarian produced estrogen levels rise throughout the proliferative phase the endometrial cells begin to rapidly divide (Young 2000). Estradiol acts through receptors located on stromal and epithelial cells, which also causes the simple tubular glands to proliferate, forming numerous glands. The proliferative

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phase is maintained until ovulation by the increasing synthesis and secretion of estrogen from the developing ovarian follicles.

The secretory phase of the uterine cycle occurs after ovulation and is characterized by progesterone and estrogen induced endometrial changes (Gray 2005). Estrogen causes progesterone receptor expression and is therefore an important regulator of progestational effects (Blake 2012). Progesterone's effects on endometrial stromal cells, also referred to as the decidual reaction, are evident in the early secretory phase. During this phase the endometrium grows up to 6 mm and the glands become highly coiled (Young 2000). Endometrial stromal glands are induced by progesterone to produce a thick, glycogen-rich secretion, which is an important nutritive factor for the embryo while vascular connections are being established with the mother's vasculature. In the absence of fertilization, the late secretory phase of the menstrual cycle shows a regression of glandular secretory activity.

In the absence of fertilization the menstrual phase of the uterine cycle begins (Young 2000). As previously mentioned, without progesterone, the endometrium can no longer be maintained and is shed during menstruation. Blood and necrotic endometrium appear in the uterine lumen, to be discharged from the uterus and out through the vagina as menstruation (Gray 2005). During this time FSH secretion is induced, causing a new cycle of follicle development and production of estrogen, which begins the process of resurfacing. Although the process of menstruation represents the end-point of the cycle of endometrial changes, the first day of menstruation also marks the beginning of a new proliferative phase.

The uterine blood supply spans from the myometrium through both layers of the endometrium and is predominantly arterial (Ross 2011). The distinguishing arrangement of the endometrium's arterial supply has essential influences on the menstrual cycle (Young 2000). Branches of the uterine artery extend through the myometrium and bifurcate into two different types of arteries, spiral arteries and straight arteries, as they extend into the endometrial layer.

The straight arteries are short and supply the stratum basalis of the endometrium (Young 2000). Spiral arteries are opposite; they are long, coiled and thick-walled. These arteries supply the surface of the endometrium giving off numerous branches that surround the stromal glands. The spiral arteries, unlike straight arteries, react to hormonal changes of the uterine cycle. The end of the secretory phase, characterized by inhibition of estrogen and progesterone secretion, causes the spiral arteries to constrict and leads to an ischemic phase. This phase immediately precedes menstruation and induces degeneration of the endometrium. The spiral artery along with lacunae and capillary beds are part of the stratum functionalis, which is shed and then reformed each month (Ross 2011).

2.5 Ectopic Endometrium

Eutopic endometrium is thought to be the tissue from which endometriosis develops. Because of this, a comparison between ectopic and eutopic endometrial tissue is important to the study of this disease. Ectopic endometrium, no matter the location, typically resembles the eutopic endometrial characteristics of proliferation and shedding. For this reason the

hormonal responses of both tissue types is presumed to be similar. However, studies have shown this is not the case and that other factors are involved with the development and regulation of ectopic endometrial lesions (Metzger 1993).

Jones et al (2009) examined the ultrastructure of ectopic peritoneal lesions using electron microscopy and immunohistochemistry. Heterogeneity was observed among the structure of endometriotic lesions suggesting non-uniformity, dissimilar to eutopic endometrium. A mix of all three cell types (squamous, cuboidal, and columnar) were observed in the excised peritoneal lesions. The most similar structural observation was seen in a biopsy taken on day 9 of this study, showing columnar glandular tissue, euchromatic nuclei, and a normal distribution of organelles. Certain homogenous features were noted in this study including a significant lack of glycogen accumulation normally seen in the second part of the menstrual cycle.

As previously mentioned, glycogen is the main product of endometrial glands, and is important in nourishing an embryo after implantation into the uterine wall. The lack of glycogen observed in these ectopic lesions may be associated with the increased estrogen levels found in endometriotic lesions (Bulun 2000).
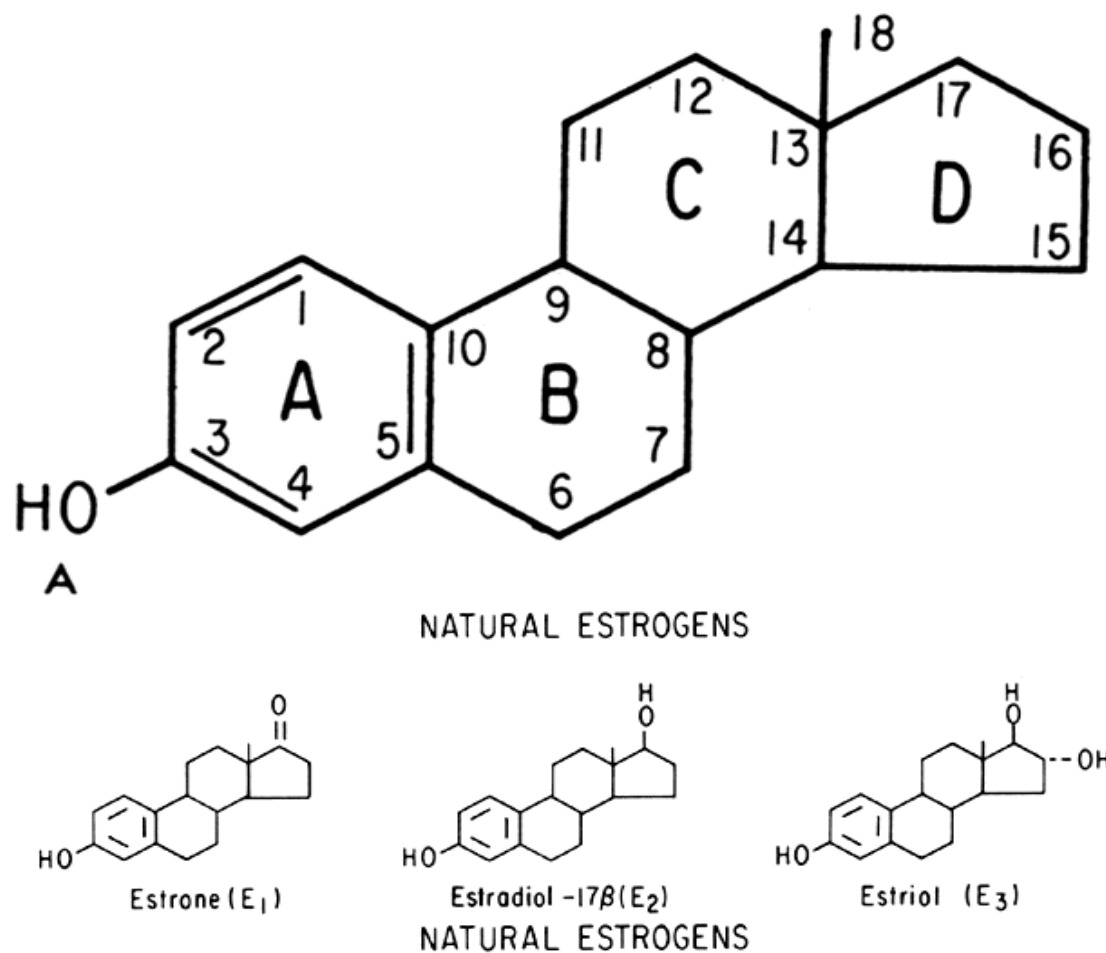
Figure 2.1 Estrogen Metabolites (From Hammond CR, Soules M: Clinical significance of estrogen metabolism and physiology. Contemp OB/GYN 11:41, 1978). Copyright © 2004 - 2011 Lippincott Williams & Wilkins, Two Commerce Square, 2001 Market Street, Philadelphia, PA 19103 U.S.A. All rights reserved.

Chapter III: Theories of Endometrial Accumulation

3.1 Overview of Endometriosis Theories

Three main theories of endometriosis etiology exist today. All three have received varying amounts of support (Vinatier 2001), however, the most accepted theory of endometriosis pathogenesis is Sampson's theory of retrograde menstruation. Because retrograde menstruation occurs in a larger percentage of menstruating women than those diagnosed with endometriosis, other factors must be involved in the pathogenesis. This theory is the basis for a lot of current research that seeks to find potential immunologic, angiogenic, and environmental factors that may allow for the implantation and development of misplaced endometrial tissue.

The other widely known theories of endometriosis etiology include, Meyer's theory of coelomic metaplasia and Halban's theory of lymphatics and vascular metastasis, which were developed around the same time (Hoffman 2011). The theory of coelomic metaplasia is based on the potential morphological changes of cells found in the ovary; because cells that make up both the ovary and the uterus are derived from the same precursor cell, the potential for metaplasia exists. Halban's theory of lymphatics and vascular metastasis suggests that endometrial tissue is taken up through the lymphatic spaces and

the vasculature, causing distant lesion formation. These theories along with related literature will be reviewed here.

3.2 Sampson's Theory of Retrograde Menstruation

In 1927 John A. Sampson, M.D. published a paper entitled "Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation" (Sampson 1927). This paper discussed this theory of endometriosis etiology based on retrograde menstruation and implantation. Sampson's theory was formed from his own observations during a series of hysterectomies performed during various stages of the menstrual cycle.

Sampson's primary theory suggests that the pathology of endometriosis occurs when endometrial tissue is shed from the mucosa of the uterus and flows back into the oviducts and into the peritoneum (Sampson 1927). Endometriosis can be induced in the baboon by endocervical canal resection, which prevents endometrial tissue from being discharged from the uterus and out through the vagina; this simulates retrograde menstruation (Dehoux 2011). This induced non-human primate model for endometriosis, discussed in greater detail in Chapter 9, supports this theory.

Sampson also suggested that endometrial tissue maybe taken into venous circulation of the uterus during menstruation, which would account for the etiology of endometrial tissue growing away from the uterus, such as on the vagina, groin, and rectum (Sampson 1927).

While retrograde menstruation has been shown to occur in up to 90% of women (Bokor 2009 and Burney 2012) with viable endometrial cells found in the fallopian tubes and in the peritoneum (Halme 1984), it would be expected to

display a higher incidence of endometriosis. This indicates that other factors besides endometrial tissue found outside of the uterine mucosa are necessary for the formation of this disease (Alford 2010).

3.3 Meyer's Theory of Coelomic Metaplasia

Meyer's theory, also commonly referred to as the theory of coelomic metaplasia, explains only ovarian and pelvic peritoneal endometriosis (Meyer 1919). This theory is established on the basis that both the germinal epithelium of the ovary, the pelvic peritoneum, and that of the uterus are derived from the same precursor cell. It infers that the germinal epithelium of the ovary and the pelvis can morph by metaplasia into endometrial tissue (Vinatier 2001). This theory is appealing due to its suggestion of the etiology of endometriosis without menstruation playing a factor. However, there are also several facts unexplained by this theory.

First, if peritoneal metaplasia explains the origin of this disease, a similar disease should be observed in males (Vinatier 2001). Coelomic mesothelium, also mesodermal epithelium, is one of three common cell sources of testes and ovarian development (Ross 2011). In males, coelomic mesothelium gives rise to Sertoli cells and the primary sex cords. As development continues, the primary sex cords differentiate into the seminiferous cords, which later develop into the seminiferous tubules, straight tubules, and rete testis. If metaplasia occurs with these precursor cells in women, it would be most likely, also be seen in men.

Secondly, coelomic metaplasia would be observed in all tissues derived from the coelomic epithelium, which include both striated and smooth muscle, the heart, blood and lymphatic vessels, the spleen, kidneys, and the adrenal

cortex (Ross 2011). Finally, if coleomic metaplasia mimics typical metaplasia, then endometriosis should be more common in older women.

While this theory makes sense, because cells of the ovary, pelvic peritoneum, and endometrial cells are both derived from the same coelomic precursor cell in the developing embryo, it has been difficult to provide scientific evidence to support it (Alford 2010). However, studies that examine benign and malignant epithelial ovarian tumors and the correlation between endometriosis may provide support for this theory. Epithelial ovarian tumors are considered to be derivatives of germinal epithelium; the presence of ovarian surface epithelium could be accounted for by coelomic metaplasia (Somigliana 2006).

3.4 Halban's Theory of Lymphatic and Vascular Metastasis

Another theory of endometriosis etiology is that of vascular and lymphatic metastasis (Vinatier 2001). Halban suggested that during the invasion of the myometrium by its mucosa, bits of the endometrial tissue are taken up into lymphatic spaces between muscle bundles of the uterine wall and are transported to superficial lymphatics. This theory suggests that distant lesions are formed by endometrial tissue being taken up by the blood supply and lymphatic vessels in the uterus and transported to other areas of the body. While this theory could potentially explain distant lesions, it does not explain the particular location of most endometrial lesions, which is on the surface of ovaries or within the abdominal peritoneum.

3.5 Conclusions

These theories of endometriosis etiology have provided researchers with a starting point, leading to some important discoveries about factors involved in

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the development of this disease. However, these theories have been difficult to support and therefore remain inconclusive.

Chapter IV: Diagnosing Endometriosis

4.1 Overview

The primary method of diagnosing endometriosis today is via
laparoscopy, which sometimes includes taking a biopsy for a histological
diagnosis (Hoffman 2011). Due to the varying degrees of endometriosis among
women there have been attempts to create a standardized classification system in
order to objectively evaluate the extent of the disease based on the amount of
ectopic endometrial tissue and the appearance of the endometriotic lesions.

The first classification system, provided by the American Fertility Society
(AFS) in 1979, was a scoring system that experimented with labeling the severity
of the disease based solely on observation (Adamson 2011). Since then there have
been two revisions of this classification systems as well as other methods of
classification. These include the ENZIAN classification system and the
Endometriosis Fertility Index (EFI), which will be discussed in this chapter.

4.2 The American Fertility Society Classification System

The AFS, later renamed the American Society for Reproductive Medicine
(ASRM), revised its classification system in 1985 and is currently the best known
and most widely used classification system for endometriosis (Haas 2013). The
revision distinguishes between superficial and invasive levels of the disease.
According to the revised classification system, there are four stages

of endometriosis: stage I (minimal), stage II (mild), stage III (moderate), and stage IV (severe). Staging is determined by five factors, including the location of the endometrial lesions, the size of the lesions, the presence of adhesions, the extent of the adhesions, and the degree of obliteration of the posterior cul-de-sac (Adamson 2010). Similar results were published in support of the AFS's revised classification of endometriosis and concluded that visual documentation of endometriotic lesions were reproducible and therefore sufficient in making a determination on the stage of endometriosis according to the American Fertility Society classification (Rock 1995).

The ASRM produced a second revision of the classification system for endometriosis in 1997 in order to update the system based on surgical findings (rASRM classification system). There were no apparent changes made to the stages defined in the 1985 classification, however, an additional description of lesion morphology (white, red, and black) was included in this revision. While the second rASRM's scoring system is the most commonly used system to determine the level of severity of endometriosis, studies have shown that this classification does not predict fertility or the severity of associated pain in individuals (Guzick 1997). This classification system also lacks assessment of deep infiltrating endometriosis. A work sheet for this classification system is shown in figure 4.1 However, both the ENZIAN system and the EFI have set out to accomplish this by making other scoring systems that measure pregnancy rates for endometriosis patients as well as developing methods in predicting pain. These scoring systems are discussed in the following sections.

In order to determine the rASRM score, numbers are given to endometriotic lesions found in the peritoneum and ovaries using points that are

to represent these lesions (Haas 2013). In addition, points are given for adhesions on the ovaries and the oviducts as well as for partial or complete posterior cul-de-sac obliteration. Each stage is scored as follows: stage I (1-5 points), stage II (6-15 points), stage III (16-40 points), and stage IV (>40 points).

4.3 The ENZIAN Classification System

Deep infiltrating endometriosis (DIE) is a special type of endometriosis that infiltrates the peritoneal surface >5 mm (Koninckx 1994). These lesions are extremely active and significantly correlated with pelvic pain (Koninckx 1991). The ENZIAN classification system was developed because the rASRM classification of endometriosis does not assess this particular development of endometriosis.

The ENZIAN classification system was established in 2005 with the intentions of supplementing the rASRM's classification of endometriosis rather than challenging it. The ENZIAN classification system attempts to provide a way to classify DIE. In February 2011 the second revision of the ENZIAN classification system was released. The revised classification system divides the retroperitoneal structures into three separate compartments as follows: Compartment A (retrovaginal septum and vagina), Compartment B (sacrouterine ligament to pelvic wall), and Compartment C (rectum and sigmoid colon) (Haas 2012). Similar to the rASRM system, the severity rating for each compartment is indicated by a grading scale. The grading system is 1-3 based on the depth of invasion: a grade of 1 will be used to indicate invasion <1 cm, grade 2 indicates invasion 1-3 cm, and grade 3 indicates invasion >3 cm. While the ENZIAN system of classification is meant to supplement the rASRM classification of

endometriosis, it is not yet internationally accepted. A worksheet for the ENZIAN classification system is shown in figure 4.2.

4.4 The Endometriosis Fertility Index

As previously mentioned, classification of endometriosis based on the rASRM classification system is unable to predict fertility in women suffering from endometriosis. The EFI staging system was created in order to effectively assess fertility outcomes based on clinical data (Adamson 2010). This clinical tool has been deemed clinically useful for patients surgically diagnosed with endometriosis attempting non-IVF conception. A study was performed using data collection and statistical analysis on 579 infertile patients in order to produce this method of staging. After creation of this fertility index, data was then collected and EFI scores were calculated on an additional 222 patients. Subsequently, the actual pregnancy rates compared with the EFI predicted rates.

The EFI score is a sum total of two separate scores: a historical factors score and a least function (LF) score. The historical components evaluated in preliminary analyses were age, duration of infertility, and pregnancy history (Adamson 1993). These determinants were also used to make up the historical factors score, while the LF score based on surgical observations was used to supplement this (Adamson 2010). The LF score is determined by the observation of dysfunction of the reproductive structures involved in reproductive processes including, the fallopian tubes, fimbria, and ovaries. The points used to calculate LF scores are 0= absent or nonfunctional, 1= severe, 2= moderate, 3= mild dysfunction, or 4= normal; these points are given by a surgeon with respect to the capability of the organ/structure to function. Scores for each structure

(fallopian tubes, fimbria, and ovaries) were given separately for each side and then combined. After all scores were given, they are summed for both the right and left side of each structure. The lowest, corresponding to the severity for the right and left of each side, are combined for the LF score. The final EFI score is then calculated by adding the historical factors score and the LF score together. The EFI surgery form is shown in figure 4.3.

AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE
REVISED CLASSIFICATION OF ENDOMETRIOSIS

Patient's Name _____ Date _____

Stage I (Minimal) - 1-5
Stage II (Mild) - 6-15
Stage III (Moderate) - 16-40
Stage IV (Severe) - >40
Total _____

Laparoscopy _____ Laparotomy _____ Photography _____
Recommended Treatment _____
_____
Prognosis _____

PERITONEUM	**ENDOMETRIOSIS**	<1cm	1-3cm	>3cm
	Superficial	1	2	4
	Deep	2	4	6
OVARY	R Superficial	1	2	4
	Deep	4	16	20
	L Superficial	1	2	4
	Deep	4	16	20

	POSTERIOR CULDESAC OBLITERATION	Partial	Complete
		4	40

	ADHESIONS	<1/3 Enclosure	1/3-2/3 Enclosure	>2/3 Enclosure
OVARY	R Filmy	1	2	4
	Dense	4	8	16
	L Filmy	1	2	4
	Dense	4	8	16
TUBE	R Filmy	1	2	4
	Dense	4*	8*	16
	L Filmy	1	2	4
	Dense	4*	8*	16

*If the fimbriated end of the fallopian tube is completely enclosed, change the point assignment to 16.

Denote appearance of superficial implant types as red [(R), red, red-pink, flamelike, vesicular blobs, clear vesicles], white [(W), opacifications, peritoneal defects, yellow-brown], or black [(B) black, hemosiderin deposits, blue]. Denote percent of total described as R___%, W___% and B___%. Total should equal 100%.

Additional Endometriosis: _____

Associated Pathology: _____

To Be Used with Normal Tubes and Ovaries

L   R

To Be Used with Abnormal Tubes and/or Ovaries

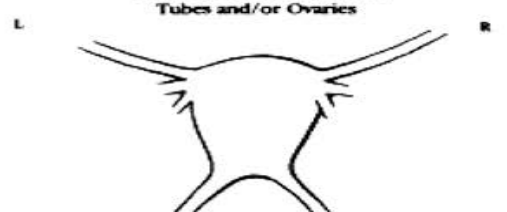L   R

Figure 4.1 "Revised American Fertility Society Classification of Endometriosis: 1985." Revised American Fertility Society Classification of Endometriosis: 1985. *Fertil Steril* 43 (3): 351-2.

Figure 4.2 The revised ENZIAN classification of endometriosis. Reprinted from Acta Obstetricia et Gynecologica Scandinavica, Dietmar Haas, Omar Shebl, Andreas Shamiyeh, Peter Oppelt, "The rASRM and the Enzian classification for endometriosis: their strengths and weaknesses," 3-7, © 2012 The Authors © 2012 Nordic Federation of Societies of Obstetrics and Gynecology, with permission from John Wiley and Sons

ENDOMETRIOSIS FERTILITY INDEX (EFI) SURGERY FORM

Endometriosis fertility index surgery form.

LEAST FUNCTION (LF) SCORE AT CONCLUSION OF SURGERY

Score		Description		Left	Right
4	=	Normal	Fallopian Tube		
3	=	Mild Dysfunction			
2	=	Moderate Dysfunction	Fimbria		
1	=	Severe Dysfunction			
0	=	Absent or Nonfunctional	Ovary		

To calculate the LF score, add together the lowest score for the left side and the lowest score for the right side. If an ovary is absent on one side, the LF score is obtained by doubling the lowest score on the side with the ovary.

Lowest Score [ ] + [ ] = [ ]
Left Right LF Score

ENDOMETRIOSIS FERTILITY INDEX (EFI)

Historical Factors			Surgical Factors		
Factor	Description	Points	Factor	Description	Points
Age			LF Score		
	If age is ≤ 35 years	2		If LF Score = 7 to 8 (high score)	3
	If age is 36 to 39 years	1		If LF Score = 4 to 6 (moderate score)	2
	If age is ≥ 40 years	0		If LF Score = 1 to 3 (low score)	0
Years Infertile			AFS Endometriosis Score		
	If years infertile is ≤ 3	2		If AFS Endometriosis Lesion Score is < 16	1
	If years infertile is > 3	0		If AFS Endometriosis Lesion Score is ≥ 16	0
Prior Pregnancy			AFS Total Score		
	If there is a history of a prior pregnancy	1		If AFS total score is < 71	1
	If there is no history of prior pregnancy	0		If AFS total score is ≥ 71	0
Total Historical Factors			**Total Surgical Factors**		

EFI = TOTAL HISTORICAL FACTORS + TOTAL SURGICAL FACTORS: [ ] + [ ] = [ ]
Historical Surgical EFI Score

ESTIMATED PERCENT PREGNANT BY EFI SCORE

EFI SCORE
9-10
7-8
6
5
4
0-3
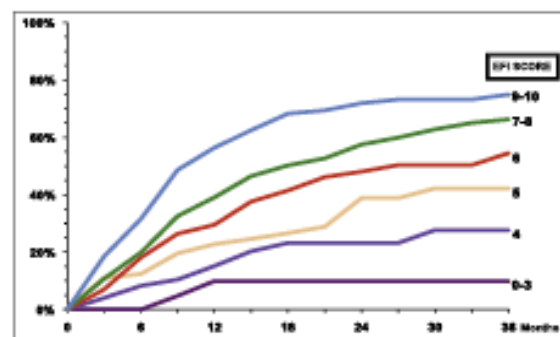
Figure 4.3 The EFI Surgery Form. Reprinted from Fertility and Sterility, Volume 94, G. David Adamson and David J. Pasta, "Endometriosis fertility index: the new, validated endometriosis staging system," 1609-1615, © 2010, with permission from Elsevier.

Chapter V: Angiogenesis

5.1 Overview

Angiogenesis, the formation of new blood vessels from preexisting vessels, is crucial to the development, implantation, and progression of endometriosis. In normal women, endometrial stromal cells release angiogenic factors, which signal the vasculature of the stratum functionalis to proliferate. Vascular endothelial growth factor (VEGF) is the most studied pro-angiogenic factor that signals angiogenesis and vascularization (Hoffman 2011). Endothelial cells are steadily experiencing shear stress or the pressure applied by blood flow. Shear stress, inflammation, and hypoxia, or oxygen deficiency, in cells induces VEGF production (Ross 2011). The role of VEGF and other angiogenic factors are among current research topics in endometriosis pathology.

Ectopic endometrial tissue requires angiogenesis for development, which suggests its potential role in endometriosis pathology. The complex process of angiogenesis begins with degradation of the basement membrane of a capillary or vein, migration and proliferation of endothelial cells, sprouting and adjoining of two vessels, and finally maturation of those vessels. In addition to VEGF, Fibroblast Growth Factors (FGFs), Platelet-derived Growth Factor (PDGF), Transforming Growth Factor-alpha (TGFα), Transforming Growth Factor-beta (TGFβ), and Tumor Necrosis Factor-alpha (TNFα) are the most significant

inducers of angiogenesis as it relates to the female reproductive system. The role of VEGF, FGF, and PDGF in the normal female reproductive tract and in endometriosis will be presented below.

5.2 VEGF, FGF, and PDGF in the normal female reproductive tract

VEGF is a secreted heparin-binding, dimeric glycoprotein that acts as a chemo-attractant to endothelial cells. It is crucial to blood vessel formation, as studies have shown that inactivation of VEGF in embryonic stem cells leads to malformation of blood vessels within the embryo (Ferrara 1996). VEGF has several isoforms encoded by a single gene (Enholm 1997). These isoforms, VEGF-A, VEGF-B, and VEGF-C, are created through alternative splicing methods and have different effects on various populations of endothelial cells (Abulafia 1999). Tumors produce elevated levels of VEGF in order to establish a vascular supply (Shweiki 1993); it is believed that ectopic endometrial tissue utilizes a similar action for survival.

VEGF-A is the most important angiogenic factor of the various isoforms and is typically studied for its role in tumor growth (Ferrara 2009). It is responsible for inducing both normal and pathologic angiogenesis and it is believed to play a central role in the growth and maintenance of endometriosis (Ferrara 2009 and Donnez 1998). VEGF-C was determined to be the ligand for the VEGF receptor expressed predominantly on venous endothelium of early embryos and lymphatic endothelium of adult tissues (Enholm 1997). This isoform of VEGF is increased in endometrium and promotes endothelial functions and vascular permeability (Xu 2013). VEGF-B, like VEGF-A and VEGF-

C, is expressed in most tissues, but its greatest concentrations are in the heart and skeletal muscle (Enholm 1997), which explains its lower effects on endometrium.

During the normal menstrual cycle the angiogenic response is structured and controlled with rapid growth of vessels during the secretory phase and arrested growth shortly after (Abulafia 1999). Changes in endometrial VEGF mRNA expression are observed during the menstrual cycle. Its expression increased three to five times from the early proliferative phase to the late secretory phase where it was maximal (Shifren 1996). VEGF mRNA was present in uterine glandular epithelial cells and stromal cells. The increase in VEGF mRNA expression by the late secretory phase suggests that vascular endothelial growth factor may impact expansion and coiling of the spiral arteries. VEGF mRNA expression is observed in many different cell types of the female reproductive system that are either involved in the process of steroidogenesis, for example, granulosa, theca, and luteal cells; or are steroid responsive, including oviductal epithelium and endometrial stromal cells. VEGF expression is hormonally regulated and up-regulated in cells that produce steroid hormones (Shweiki 1993). Overexpression of VEGF leads to pathology, including cancers and endometriosis, which similarly require a blood supply for cell survival.

Fibroblast growth factors (FGFs) are secreted proteins produced by vascular endothelial and smooth muscle cells. FGFs and their receptors are regulators of angiogenesis, steroidogenesis, proliferation, differentiation, migration, and cell-survival (Brooks 2012). FGFs are heparin-binding growth factors, while their receptors are part of the tyrosine kinase receptor family (Chaves 2012). After various studies identified expression of FGFs in reproductive tissue, further experimentation demonstrated the involvement of

FGFs in the physiological regulation of reproductive processes and ovarian function.

FGF-2 is the most studied and characterized growth factor of the FGF family and is involved in the survival of reproductive cells. Similar to VEGF, FGF-2 is an angiogenic growth factor present in both normal and abnormal endometrium. This factor is important not only for angiogenesis in general, but also specifically for angiogenesis of endometrial tissue (Möller 2001). A study by Zhou et al (2005) demonstrated that FGF-2 could maintain oocyte survival in culture, but did not seem to have an effect on growth. A second study also demonstrated that FGF-2 prevents granulosa cell apoptosis in an in vitro study with rat granulosa cells (Tilly 1992). Dysregulation of the FGF/FGFR pathway and overexpression of FGFR have been correlated with developmental disorders, cancers, and endometriosis.

Another factor involved in angiogenesis and therefore studied in correlation with endometriosis pathology is Platelet-derived growth factor. PDGF is a protein released by platelets that induces proliferation of fibroblasts (Campbell 2005). The binding of PDGF with its receptor, part of the tyrosine kinase receptor family, initiates a signal transduction pathway enabling the connective tissue fibroblasts to divide. It is possible that a combination of all three of these angiogenic factors is necessary for the development and maintenance of endometriosis.

5.3 Vascular Endothelial Growth Factor in Endometriosis

In order for endometriosis to progress, endometrial tissue must implant in an extra-uterine location and obtain a blood supply in order to grow. Active

endometrial lesions potentially activate angiogenesis within and around adjacent tissue (Abulafia 1999). Increased levels of angiogenic factors, including VEGF in the peritoneal fluid of women with endometriosis compared to women without endometriosis have been observed (McLauren 1996). This finding is consistent with the endometriosis requirement for angiogenesis and suggests pathology due to the overexpression of angiogenic factors. It was also observed that VEGF concentrations were significantly higher during the proliferative phase than the secretory phase. Taylor et al (1997) showed that increased levels of VEGF and IL-8 in peritoneal fluid have positive correlation with the number of active endometriosis lesions. The same study also demonstrated a fast-acting and direct effect of E2 on VEGF mRNA and protein synthesis in endometrial stromal cells. The addition of 10 nM E2 to endometrial stromal cells caused a 50% increase in VEGF secretion.

As previously mentioned various isoforms of VEGF are responsible for different functions and properties of angiogenesis. A study by Xu et al (2013) found increased levels of VEGF-A, VEGF-B, and VEGF-C proteins in the eutopic and ectopic endometrium of an endometriosis nude mouse model. However, VEGF-A mRNA in ectopic endometrium and VEGF-C in both eutopic and ectopic endometrium were found to be significantly higher in patients with endometriosis than controls. It was also determined that VEGF-C significantly enhanced development and angiogenesis of endometriosis.

A study by Laschke et al (2007) determined that COX-2 inhibitors inhibit VEGF-mediated angiogenesis. COX-2 is an enzyme responsible for producing prostaglandins involved in inflammation, pain, and fever (Lehninger 2008). Two studies demonstrated that COX-2 inhibition prevents implantation of

endometrium at ectopic sites, which is a vital step in the survival of endometrial tissue outside of the uterus, and ultimately development of endometriosis. COX-2 inhibitors and their effects will be discussed in more detail in Chapter 10.

5.4 Fibroblast Growth Factor in Endometriosis

FGF-2, as previously mentioned, is an angiogenic growth factor present in both normal and abnormal endometrium (Bourlev 2006). This factor is important not only for angiogenesis in general, but also specifically for endometrial angiogenesis.

FGF-2 induces angiogenesis by binding to one of its receptors FGFR-1 or FGFR-2 (Möller 2001). Data shows that both receptors of FGF-2 are present in human endometrium (Ferriani 1993). Specifically, both receptors were expressed in arteries, but not in veins during the late secretory phase. However, the expression of FGFR-2 in endometrial glands was observed to be unrelated to menstrual cycle phase (Möller 2001).

FGF-2 has also been detected in both serum and the peritoneal fluid of patients with endometriosis (Bourlev 2006). Individuals with surgically confirmed endometriosis compared with controls had observed higher concentrations of FGF-2 during both the proliferative and secretory phases of the menstrual cycle. Also, the FGF-2 levels observed in the peritoneal fluid were significantly higher than in serum.

5.5 Platelet Derived Growth Factor in Endometriosis

PDGF induces its greatest effects on endometrial luminal and glandular epithelial cells during the proliferative phase and lesser effects in the secretory

phase (Chegini 1992). PDGF induces cell division of endometrial stromal cells suggesting a modulatory role on endometrial cell growth and differentiation.

Surrey et al (1991) used proliferative phase stromal cells isolated from the endometriotic lesions biopsied from individuals with endometriosis. These cells grown in culture were used to model the stromal composition of ectopic endometrium. PDGF and physiological E2 concentrations caused a significant increase in proliferation of the endometrial stromal cells. This suggests that PDGF may play an important role in the development or maintenance of endometriosis.

5.6 Conclusions: A Cooperative Role for VEGF, FGF, and PDGF

Data support cooperation between VEGF, FGF, and PDGF in the ability of ectopic endometrium to establish and maintain a new blood vessel formation. A study by Laschke et al (2006) determined that the combined inhibition of VEGF, fibroblast growth factor, and platelet-derived growth factor effectively suppressed angiogenesis and vessel maturation of endometrial grafts. The combined inhibition was more effective than inhibition of VEGF alone. Combined inhibition significantly reduced the size of the microvascular network and decreased micro-vessel density. This suggests a cross talk between VEGF, FGF, and PDGF in the establishment of microvascularization of endometriotic lesions. Thus, the ability of new endometriotic lesions to develop may strongly depend on the microenvironment concentrations of these angiogenic factors, and their inhibition may be one approach to controlling new lesion growth.

Chapter VI: Immunology of Endometriosis

6.1 Immunology Overview

The immunology of endometriosis has been explored as a potential factor in the pathology of this disease. Immune cells have been shown to play a critical role in allowing refluxed endometrial cells to implant and grow rather than be eliminated. The presence of lymphocytes, natural killer (NK) cells, macrophages, and mast cells in endometriotic lesions suggests the influence of these immune cells on this disease. T lymphocytes and macrophages are the two major leukocyte populations found in endometrial tissue (Osuga 2011). A brief review of these immune system cells will give a better understanding of their role and importance as it pertains to endometriosis. This chapter discusses the potential role of immune cell populations in the pathology of endometriosis.

6.2 Cells of the Immune System

The predominant cells of the immune system are lymphocytes, antigen-presenting cells, and effector cells (Abbas 2012). There are two major types of lymphocytes that are distinguished based on their functions and how they recognize foreign substances. B-lymphocytes, the major components of humoral immunity, account for 20-30% of the circulating lymphocyte population (Ross 2011). These cells are produced in the bone marrow to recognize specific antigens, differentiate into plasma cells, and synthesize antibodies.

T lymphocytes, which make up a majority of the circulating lymphocyte population, characterize cell-mediated immunity (Abbas 2012). These cells recognize intracellular microbes and either directly induce apoptosis of infected cells, secrete cytokines, or regulate the immune response. Three different subpopulations of T lymphocytes serve different functions: T helper cells, cytotoxic T cells (CTLs), and regulatory T cells (T regs). T cells are educated in the thymus to recognize and destroy foreign antigens (Ross 2011). The immune system communicates by sending out signals that lead to activation, recruitment, or allow for other physiological effects to occur. These signals are typically released from immune cells as cytokines or chemokines. Secreted cytokines recruit other cells such as macrophages and neutrophils, which respond by phagocytosing infected cells.

NK cells, derived from the same precursor cell as T and B cells, are a third subset of the lymphocyte population that participates in the body's defense against viruses and intracellular pathogens (Abbas 2012). These cells are similar to cytotoxic T cells, but their cell surface receptors are different than those found on T and B cells.

Macrophages are antigen-presenting cells (APC) of the mononuclear phagocytic system (MPS), which is a group of cells in which the primary function is to phagocytose foreign antigens (Abbas 2012). All cells of MPS are derived from a common precursor cell found in the bone marrow. Two major cell lineages are derived from a common precursor cell in the bone marrow called hematopoietic stem cells (HSCs). HSCs can become monocytes, which ultimately differentiate into macrophages and are activated in multiple tissues in the body. T helper cells that recognize antigen displayed on the cell surface by antigen

presenting cells activate macrophages; this causes the T helper cells to release cytokines that then activate the macrophages (Ross 2011). Macrophages are recruited to a site of infection by chemokines produced by T cells. T cells also secrete cytokines that induce other cells to release chemokines for macrophage recruitment.

A second type of APC is a dendritic cell (DC) (Abbas 2012). DCs play a crucial role in innate immunity and activation of naïve T cells. These cells are characterized by long projections, referred to as dendritic processes, of their membrane. DCs mature in response to a cytokine called Flt3 ligand. They function by recognizing receptors expressed by microbes, phagocytosing the foreign pathogen, and presenting the antigen to circulating T cells which are then activated and can perform its functions.

Mast cells, similar to macrophages in origin, are derived from bone-marrow cells present in mucosal epithelium (Abbas 2012). Mast cells contain granules in their cytoplasm filled with cytokines and histamine. Mature mast cells are not typically found in peripheral blood circulation, but are usually found within tissues near small blood vessels and nerves. Products released by microbes or via an antibody-dependent mechanism activate mast cells. When activated these cells release histamine, which is a vasoactive amine that causes vasodilation and increased capillary permeability. Mast cells also synthesize prostaglandins and cytokines, such as TNFα Because mast cells are common near blood vessels and nerves, the release of their contents causes changes in the blood vessels that lead to acute inflammation. Interleukin 12 (IL-12), a cytokine involved in CD4+ cell differentiation, is secreted by both macrophages and DCs.

The binding of this cytokine to its receptor on the surface of NK cells and T cells induces interferon gamma (IFNγ) synthesis and secretion.

6.3 Immune Cell Involvement in the Menstrual Cycle

Northern et al (1994) conducted a study to determine peripheral lymphocyte concentrations in normal menstruating women and found that total white blood cells (WBCs) significantly increased in the blood during the day and peak around 2 pm on day six of the menstrual cycle. These levels then declined to their lowest point overnight. Differences were observed in cell counts during the normal menstrual cycle: white blood cells and B cells were significantly higher during the proliferative phase, whereas NK cells were significantly higher during the secretory phase. In general, this study found that daily total WBCs were lowest in the morning and peaked by 2 pm.

Lee et al (2010) conducted a study to see if the lymphocyte populations in peripheral blood varied during the menstrual cycle and found that all three lymphocyte populations fluctuated in response to ovarian phase. T cells were increased in the follicular phase compared to the luteal phase and NK cell levels and NK cell toxicity were significantly higher during the luteal phase than the follicular phase. While B cell concentrations changed during the menstrual cycle, these changes were not significant.

6.4 Lymphocytes in Endometriosis

Current research suggests that abnormalities in the functions of the lymphocyte populations could be responsible for the pathology of this disease. As previously mentioned there are three subsets of the T lymphocyte population: CTLs, T helper cells, and T regs. Inhibition of the functions of these populations

has been observed in women with endometriosis as well as in surgically induced endometriosis animal models. Current research demonstrates that cytotoxic T lymphocytes that are unresponsive to autologous endometrium are present in endometriosis. In a study performed by Dmowski et al (1981) the cytotoxicity of T lymphocytes against autologous endometrium was observed to be significantly decreased in women with endometriosis. Decreased proliferation of lymphocytes in response to autologous endometrial cells was seen also in women with endometriosis (Helvacioglu 1997).

A second method by which endometrial cells have demonstrated an ability to evade CTL immune surveillance is through cytokine-mediated expression of the transmembrane protein Fas Ligand (FasL). FasL belongs to the TNF family and induces apoptosis by binding to its receptor on adjacent cells. FasL expression by endometrial stromal cells is caused by the cytokine IL-8 and the chemokine CCL2 (Osuga 2011). Secretion of CCL2 by endometrial cells up-regulates FasL mediated apoptosis in T-lymphocytes. This suggested method of evasion was further supported by two studies by Selam B et al (2002, 2006) that demonstrated that IL-8 and CCL2 were increased in the serum and peritoneal fluid of women with endometriosis.

CD4+ and CD8+ T cell abundance or activity is altered in endometriosis. A few studies have shown a decreased CD4:CD8 ratio in endometriotic peritoneal fluid (Osuga 2011). CD4+ T helper cells also have decreased activity in women with endometriosis. While there were high levels of CD4+ T lymphocytes there were decreased levels of activated CD4+ T lymphocytes indicating that the activation of these cells has been suppressed in women with endometriosis. A study by Lee et al (2005) (Lee 2005) showed that THP1 cells,

cells of the monocyte cell lineage, exhibited decreased expression of MHC class II when co-cultured with endometriotic peritoneal fluid. MHC class II is a cell surface protein that plays a fundamental role in antigen recognition by CD4+ T cells (Abbas 2012). This protein is crucial in T cell activation and therefore its decreased expression associated with endometriosis could explain the decreased cytotoxicity of CD8+ T cells and the decreased activation of CD4+ T helper cells.

Regulatory T cells, a recently discovered subpopulation of T lymphocytes, are important in suppressing the immune system (Osuga 2011). In women without endometriosis there is a significantly decreased level of regulatory T cells in peripheral blood circulation during the secretory phase of the menstrual cycle, while this was not observed in women with endometriosis. This suggests that the unsuppressed levels of T regs prevent the recruited immune cell populations from clearing the shedded endometrial tissue and therefore allow endometriotic tissue survival and implantation.

The suggested role of B cells in the pathology of endometriosis is via secretion of autoantibodies. Badawy et al (1989) showed that the amount of IgG and IgA produced by peritoneal B cells was increased in women with endometriosis. This suggests an increased B cell activity in endometriosis. A second study by Odukoya et al (1995) also supports this idea of increased B cell activity. This lab observed increased serum concentrations of soluble CD23, a low affinity receptor for IgE that is produced by activated B cells, in patients with endometriosis.

6.5 Natural Killer Cells in Endometriosis

Natural Killer (NK) cells are responsible for tumor rejection as well as the removal of infected cells (Osuga 2011). As previously mentioned NK cells function by releasing small granules that induce apoptosis. Oosterlynx et al (1991) showed that in peripheral blood NK cells can destroy endometrial cells, which suggests the NK cell's role in clearing refluxed endometrial cells in the peritoneum. NK cell activity was reduced in women with endometriosis (Viganò 1991 and Garzetti 1993), which suggests that decreased NK cell cytotoxic activity potentially enables endometriosis development. Also, the reduced function of NK cell activity was correlated with the severity of endometriosis.

6.6 Macrophages in Endometriosis

Macrophage migration inhibitory factor (MIF), also called Glycosylation-inhibiting factor, is a cytokine secreted by macrophages. MIF is an important immune system regulator and functions in inhibiting macrophage migration (Abbas 2012). MIF has also been shown to influence angiogenesis and cause inflammation. Increased levels of MIF were observed in the eutopic endometrial tissue of women with endometriosis. There were also significantly increased levels of MIF found in circulation and local peritoneal fluid and in active endometrial lesions.

A study performed using an MIF inhibitor called (S, R) 3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic methyl ester (ISO) decreased the number of endometriotic lesions observed in an in vivo animal model for endometriosis. The effects of ISO on apoptosis was also examined by looking at anti-apoptotic and pro-apoptotic factors. Bax, an important pro-apoptotic factor,

was shown to be upregulated in endometriotic lesions treated with ISO; whereas there was a significant decrease in protective Bcl2 expression in lesions also treated with ISO.

6.7 The Role of Inflammatory Cytokines in Endometriosis

Various cells of the immune system secrete inflammatory cytokines such as TNFα, IL-6, and IL-12. Because endometriosis is characterized by pain associated with inflammation, the role of pro-inflammatory cytokines in the development of endometriosis has been examined.

TNFα mRNA expression is increased in the endometrium and peritoneal fluid of women with endometriosis compared to healthy controls (Kyama 2006). Kim et al (2013) looked at the effects of both sex steroids and TNFα on a specific isoform of the Pak family, Pak4. Paks, also called P21-activated kinases, are a family of serine/threonine kinases that regulate cellular activities, such as apoptosis, cell motility, proliferation, and steroid receptor signaling (Lodish 2008). Using siRNA-mediated knock down of Pak4 in endometrial glandular and stromal cells, Pak4 was shown to be responsible for cell motility in invasion assays. Treatment of endometrial glandular tissue with 10 ng/mL and 25 ng/mL of TNFα led to a significant increase in Pak4. Also, treatment of endometrial stromal cells with progesterone led to a significant decrease in Pak4 levels. Pak4 was higher in the eutopic endometrium from patients with endometriosis and did not vary with menstrual cycle, unlike control patients that had lower Pak4 levels during the secretory phase coincident with higher serum progesterone. This latter finding is consistent with higher Pak4 levels being associated with the endometriosis progesterone-resistant phenotype.

Increased concentrations of IL-6 were also detected in the peritoneal fluid of women with endometriosis (Bersinger 2012). No fluctuations of these concentrations were observed throughout the menstrual cycle. IL-12 concentrations were also increased, in endometriosis however, levels were significantly higher in the secretory phase of the uterine cycle. A summary of the cytokines mentioned in this section can be found in Table 6.1.

6.8 Conclusions

The role of the immune system in endometriosis pathology, while not concrete is evident. Immune cell evasion and dysfunction are both linked to the ability of endometrial cells to implant and develop ectopically. Most cells of the immune system communicate through chemical signals, causing up and down-regulation of other immune system secretions and effects. This suggests a combined contribution of immune cells and various cytokines to endometriosis pathology, which makes studies identifying the role of concurrent immune factors critical.

Cytokine	Effect	Presence in Endometriosis	Location	Citation
IL-6	Pro-inflammatory cytokine	Increased in women with endometriosis	Peritoneal fluid	Bersinger 2012
IL-12	Pro-inflammatory cytokine	Increased during the secretory phase of the menstrual cycle in women with endometriosis	Peritoneal fluid	Bersinger 2012
TNFα	Pro-inflammatory cytokine and increases Pak4 levels	Increased in women with endometriosis	Endometrium and peritoneal fluid	Kyama 2006 Kim 2013

Table 6.1 Summary of Cytokines involved in Endometriosis

Chapter VII: Environmental and Dietary Factors

7.1 Overview

Both environmental contaminants and dietary factors have been postulated to contribute to endometriosis. This chapter discusses current research that potentially links environmental toxicant exposure and dietary factors to the development and progression of endometriosis.

Humans are constantly exposed to environmental toxins, particularly through the diet, which could potentially disrupt physiological processes possibly causing endometriosis (White 2009 and Bellelis 2011). Environmental toxins typically remain in the body for long periods of time due to their long half-life and can over time build up. This accumulation is due in part to the lipophilic nature of many of the environmental contaminants, which causes them to associate with and be stored in adipose and lipid rich tissues (White 2009). Environmental contaminants are being studied as potential endometriosis risk factors because of their ability to act as endocrine disruptors by altering steroid synthesis or hormone receptor function, disrupting immune function, and inhibiting reproductive function by epigenetic modifications (Bruner-Tran 2010).

Dioxins like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are environmental contaminants and have been studied in relation to endometriosis. TCDD, the structure of which can be seen in figure 1.1, is one of the most well

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studied chemical agents, because of its highly toxic nature (White 2009). In addition to dietary ingestion of environmental contaminants, other dietary constituents potentially contribute to endometriosis. Theories regarding dietary influences on endometriosis include roles for dietary fatty acids, fiber and phytoestrogens (Parazzini 2013).

7.2 TCDD

TCDD belongs to a family of chlorinated hydrocarbons commonly referred to as dioxins (White 2009). These fat-soluble toxicants resist degradation and therefore accumulate in the food supply. This explains why ingestion of contaminated food is the primary method of exposure.

TCDD typically enters the environment as an industrial by-product or from incineration (White 2009). TCDD has as estimated half-life of 11.5 years, which explains the ability of this chemical to easily accumulate in the body upon consumption of contaminated food. TCDD was first looked at for its potential role in endometriosis when a primate colony developed a high incidence of this disease following chronic dietary exposure of TCDD. TCDD levels are typically high in breast milk (Rier 2002). It has been reported that very high levels of TCDD are found in the breast milk of Belgium women, which interestingly has one of the highest endometriosis rates of any country.

Current research has sought to find steroid-mediated endometrial tissue functions in which exposure to TCDD may alter or inhibit activity (Bruner-Tran 2008). Thus far research supports a link between exposure to TCDD and the progesterone-resistant endometrial phenotype (Bruner-Tran 2010). As previously mentioned, retrograde menstruation is the most accepted theory of how

endometrial tissue makes its way into the peritoneal cavity, but it is the ability of the endometrial fragments to invade the peritoneal wall that determines the survival and growth of the endometrial tissue at ectopic sites; TCDD may influence this event. Metalloproteinases (MMPs) are enzymes needed for tissue invasion. TCDD in combination with estradiol has been shown to increase MMP expression and activity by endometrial stromal cells, providing a mechanism for TCDD promotion of endometriosis (Yu 2008).

TCDD has a high affinity for the aryl hydrocarbon receptor (AhR), which is an orphan nuclear receptor that is considered to initiate the toxic effects of these compounds (Bruner-Tran 2008). Unbound AhR receptors, found in the cytosol, are associated with a complex of chaperone proteins. These receptor complexes are expressed in both endometrial and immune cells. The binding of either dioxin or dioxin-like compounds to AhR causes the chaperone proteins to dissociate and enables the AhR to enter the nucleus. In the nucleus, the AhR rapidly forms a heterodimeric complex with an aryl hydrocarbon nuclear translocator (ARNT) protein. This process is shown is Figure 7.1. TCDD activation of the AhR leads to pro-inflammatory chemokine and cytokine production (Bellelis 2011). TCDD potentially leads to the development of endometriosis by inhibiting the anti-inflammatory effects of progesterone. It has also been shown to function by increasing levels of specific interleukins and by activating enzymes like aromatase, which would in turn increase estrogen (Rier 2002).

TCDD in combination with estrogen and progesterone has been observed to induce endometriosis implantation and growth in animals. In a murine model of endometriosis immunocompromised athymic nude mice were used to implant

endometrial tissue from normal women and women with endometriosis (Bruner-Tran 2008). The human tissue exposed to TCDD showed a higher amount of endoglin staining, a marker for cell proliferation, and exhibited a higher microvascular density at the peritoneal invasion site than the human tissue exposed to only estrogen and progesterone. In addition, many neutrophils were found in the endometrial stroma of the TCDD-exposed tissue. While low levels of neutrophils present in the lumen of endometrial glands is a normal occurrence in human endometrium during the secretory phase, increased neutrophil presence may be related to endometriosis pathology, due to their associations with early stages of inflammation and their production of the pro-angiogenic factor VEGF.

TCDD has been shown to cause the progesterone-resistant phenotype seen in women with endometriosis (Bruner-Tran 2008, 2010). Two isoforms of progesterone receptor (PR), PR-A and PR-B, are present in endometrial tissue (Kim 2013). Estrogen typically increases both PRs in normal endometrium. In endometriotic tissue PR-A levels are lower than eutopic endometrium, and PR-B is not detectable (Attia 2000). TCDD is suspected of altering the PR isoform proportions expressed.

PR-B isoform is the primary mediator of anti-inflammatory effects induced by progesterone (Osteen 2002), however PR-A, which is a truncated form of the PR, can dominantly suppress PR-B responses (Vegeto 1990). In the progesterone-resistant phenotype associated with endometriosis patients, high levels of PR-A expression relative to PR-B are seen in both eutopic and ectopic endometrial tissue (Attia 2000). Bruner-Tran et al (2010) using a murine model observed that in utero and prepubertal TCDD exposure causes infertility,

inability to sustain pregnancy to term, and a significant decrease in PR. This was observed for several generations of the TCDD-exposed mice indicating, a possible epigenetic transmission or specific mutations.

Another study produced similar results using adult endometrial stromal cells (Igarashi 2005). PR-B:PR-A ratios were significantly lower in endometrial stromal cells in women with endometriosis than in normal endometrial tissue from control patients. The same study exposed normal endometrial epithelial and stromal cells to the toxicant TCDD and observed a decrease in PR-B expression relative to PR-A.

7.3 Dietary Factors

Exposure to environmental toxins via ingestion of contaminated food is one way that diet potentially influences endometriosis pathology (Bellelis 2011). Normal dietary constituents themselves may play a role in endometriosis development or symptoms. Some theories are present regarding dietary influences, but most correlative data is from individual case reports and reviews of epidemiological findings do not consistently support any single dietary factor association with endometriosis (Parazzini 2013). Two topics worth noting are a potential influence of dietary fatty acids and modulation of estrogen content or action.

Dietary fatty acids, found in various oils, serve as the substrate for many prostaglandins including prostaglandin-E2 (PGE2) and prostaglandin-F2alpha (PGF2α). PGE2 and PGF2α are pro-inflammatory eicosanoids that may increase uterine contractions and cause pain or dysmenorrhea, a frequent symptom of endometriosis (Bellelis 2011). Peritoneal explants with endometriotic lesions

produce higher PGE2 and PGF2α than surrounding normal peritoneum indicating the lesions were the likely source of the prostaglandins (DeLeon 1988). Elevated PGE2 in the peritoneal cavity of individuals with endometriosis, was found to be attributed to both the macrophage population and ectopic endometrial cells (Sacco 2012). In addition, along with PGE2's inflammatory effects, increased VEGF was found in both the peritoneal fluid and lesion implants of individuals with endometriosis (Li 2005).

PGE2 has also been shown to play a potential role in endometriosis-infertility (Lee 2011). Sperm treated with concentrations of PGE2 similar to those observed in women with endometriosis showed a decline in fertility caused by decreased rates of acrosome reaction, which occurs when necessary enzymes for penetration of the zona pellucida are released by the sperm upon contact with the egg (Ross 2011); and oocyte penetration.

As mentioned previously, endometriosis is an estrogen-dependent disease. Altering the level of circulating estrogens can influence endometriosis pathology. Kaneda et al (1997) demonstrated in women that a diet containing low fiber showed increased estrogen concentrations and therefore low fiber intake could potentially be involved in endometriosis risk. Unfortunately, there is a lack of support for this idea, as data on crude fiber intake and endometriosis risk do not show a consistent association (Parazzini 2013).

Another way estrogen action and thus endometriosis could be affected by diet is by the intake of phytoestrogens like those found in soy products. Soybeans contain isoflavones with estrogen-like properties. Some of these molecules act as weak estrogen agonists and they may even compete with

endogenous estrogen for the estrogen receptors thereby lowering the effects of endogenous estrogens. Two components of soy, genistein and puerarin, have been investigated using animal models of endometriosis. These soy components led to a reduction of estrogen concentrations, estrogen receptor levels, and estrogen production (via aromatase) and in turn reduced size and weight of endometriotic lesions (Yavuz 2007 and Chen 2011).

7.4 Conclusions

While these environmental toxins and dietary factors have been studied in relation to endometriosis development, there is not sufficient evidence to conclude a major role in endometriosis etiology. One reason why the correlation of chemical toxicant exposure to the ultimate development of endometriosis is difficult to assess may be that exposure in adults has less of an impact than early life exposure (Bruner-Tran 2008). It is suggested that toxicant exposure during fetal life and early development are more relevant to disease advancement due to the high sensitivity to these disruptors during this critical time in development. Determining the influence of natural dietary products on endometriosis development or progression needs long-term case-controlled studies to draw firm conclusions.
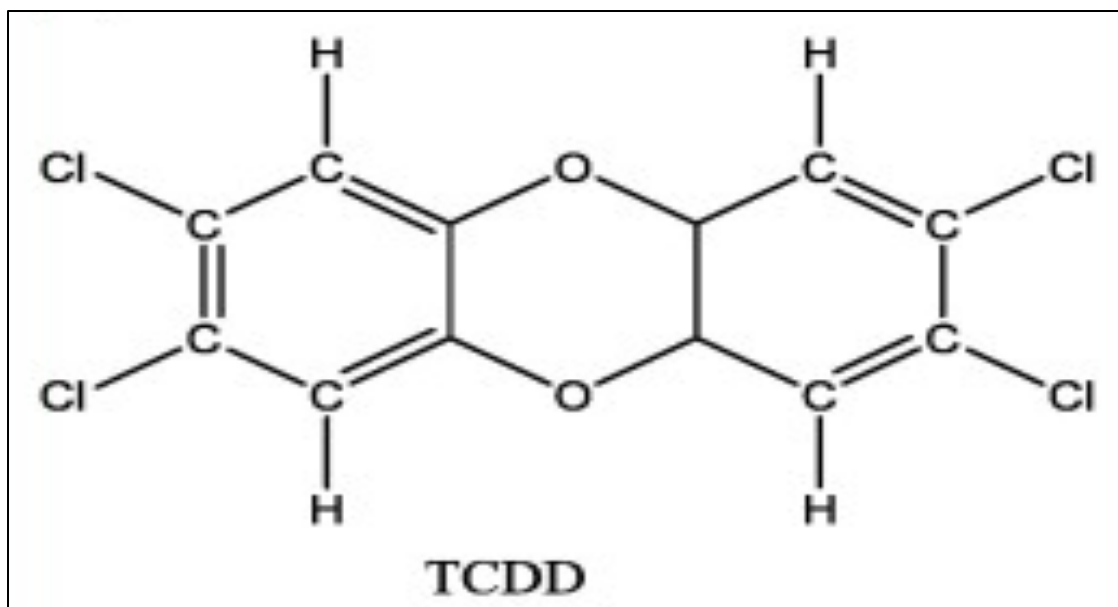
Figure 7.1 Chemical Structure of TCDD. Reprinted from Chemosphere, Volume 91, Wenxiao Pan, Yuanyuan Qi, Ruoxi Wang, Zhe Han, Dongju Zhang, Jinhua Zhan, "Adsorption of TCDD with 1-butyl-3-methylimidazolium dicyanamide ionic liquid: A combined molecular dynamics simulation and quantum chemistry study," 157-164, © 2013, with permission from Elsevier.
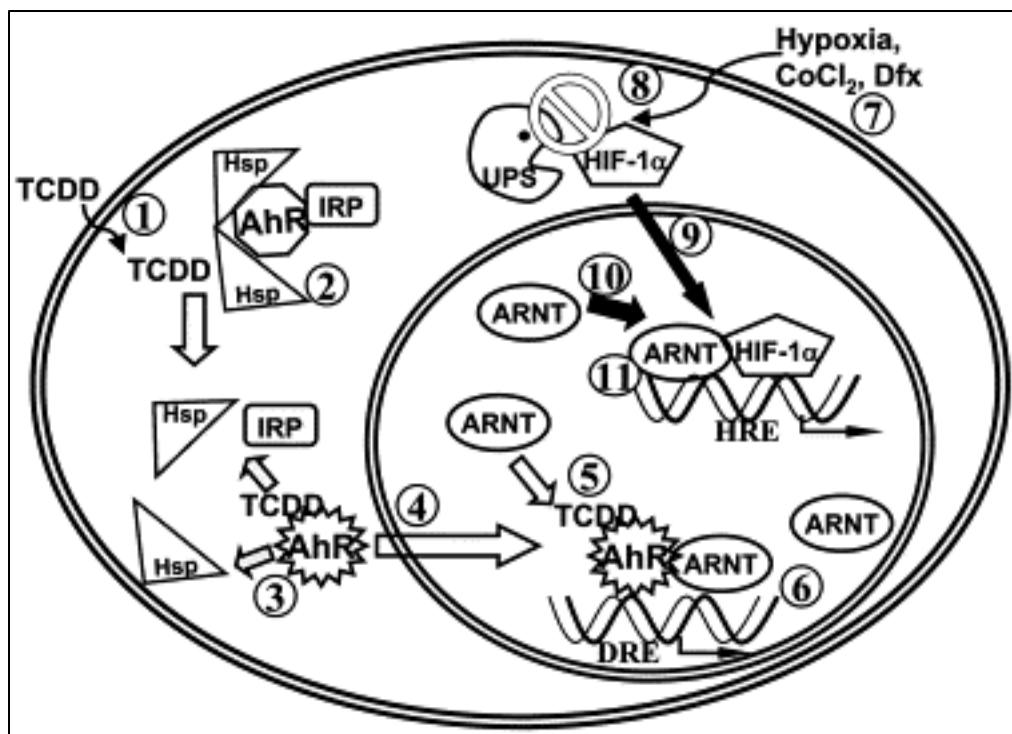
Figure 7.2 The Proposed Mechanism of action of the dioxin TCDD. Reprinted from Environmental Toxicology and Pharmacology, Volume 10, Minghua Nie, Alan L. Blankenship, John P. Giesy, "Interactions between aryl hydrocarbon receptor (AhR) and hypoxia signaling pathways," 17-27, © 2001, with permission from Elsevier.

Chapter VIII: Genetic Influence and Polymorphisms

8.1 Overview

Evidence suggests that inheritance patterns and genetic variation play a potential role in endometriosis pathogenesis (Hoffman 2011). Various mutated genes that have been passed down are currently being investigated for their role in this disease. Genetic mutation is responsible for inherited disease in humans (Lodish 2008). DNA polymorphisms are variations within the DNA sequence and can be followed throughout generations. Polymorphisms represent normal variation in a gene's sequence at a particular site. Unlike mutations, polymorphic genes are not directly linked to disease, but they can influence an individual's predisposition for a disease.

Single nucleotide polymorphisms (SNPs) are the most frequent genetic variation and are single points in the genomic sequence in which one large percentage of the population has one nucleotide and another large percentage has another (Alberts 2002). If two human genomes taken from the world's population were sequenced they would differ at approximately 2.5×10^6 points or about 1 per 1300 nucleotide pairs. Known sites of the human genome in which there is a high probability that the genomes of two people will differ are referred to as polymorphic sites. There are two approaches currently used to find correlations between particular polymorphic genes and endometriosis including

sibling-pair linkage analysis and gene expression analysis via microarray technology (Hoffman 2011). This chapter discusses several various polymorphisms involving genes that regulate inflammation and immune response, estrogen receptors, and cell cycle regulation. There are many polymorphism studies that exist to date and new studies are constantly being published. This chapter will attempt to show a range of polymorphic genes that have been identified in various populations around the world.

8.2 Specific Polymorphisms and Endometriosis

8.2.1 NFκB

NF-κB is a transcription factor that is important in inflammation and the immune response (Voet 2011). It is found in the cytosol of cells in an inactive state when it is bound to an inhibitor IκBα that prevents it from traveling into the nucleus. However, when pro-inflammatory cytokines such as TNFα and IL-1 bind to cell surface receptors they initiate a signal transduction pathway that ultimately leads to phosphorylation and dissociation of IκBα. This enables NF-κB to translocate to the nucleus and activate transcription of its target genes.

An insertion or deletion in the promoter region of the gene encoding the transcription factor NF-κB functions by decreasing the amount of RNA transcribed and in turn the amount of protein produced (Bianco 2012). In the Brazilian population, the insertion of the sequence ATTG causes a decrease in the production of NF-κB, which is positively correlated with moderate to severe endometriosis and infertility.

8.2.2 Estrogen receptors alpha and beta

Because endometriosis is an estrogen-dependent disease, genes that code for estrogen receptors alpha and beta (ERα and ERβ) are being studied. ERα and ERβ are similar, but have different mechanisms of action, slight variations in their gene and protein structures, and are encoded by different genes on separate chromosomes (Berne 2004). Both estrogen receptors have similar DNA-binding domains, but the ligand binding domains are only 55% homologous in sequence. They are also found in different locations. ERα is mostly found in uterine endometrium, whereas ERβ is typically found in granulosa cells and osteoblasts. Both receptors have the same affinity for estradiol, but varying affinities for other forms of estrogen. Estradiol has different actions in various tissues depending on the presence of a single receptor form or the ratio between the two-receptor forms.

Several studies examined ERα gene polymorphisms. In a Chinese population, a dinucleotide TA repeat (genotype E-14 repeats) in the ERα promoter was associated with endometriosis incidence (Hsieh 2005). In a Japanese population the PvuII polymorphic locus showed an increased incidence of the PP genotype with endometriosis (Kitawaki 2001). However, Sato et al (2008) examined additional intron 1 and exon 1 polymorphisms in the Brazillian population and concluded that neither of these additional sites correlated with increased risk of endometriosis in this population.

A study by Silva (2011) looked at the RsaI gene polymorphism in the ERβ gene. A higher frequency of the heterozygous AG polymorphism was discovered in individuals with endometriosis compared to patients without endometriosis.

8.2.3 p53

The transcription factor p53 is a tumor suppressor that oversees genome integrity (Voet 2011). It plays a critical role in arresting cells that have damaged DNA and preventing destructive transformation of cells. In response to damage this factor increases the ability of DNA to activate the transcription of specific genes that respond to and fix damaged DNA (Lodish 2008).

Two studies examined p53 gene polymorphisms and the association with endometriosis risk. Ribeiro Júnior et al (2009) studied the Arg72Pro polymorphism in Brazilian women and found that the proline allele was significantly associated with intense pain and was more frequent in individuals with endometriosis. A second study by Govatati et al (2012) did not find an association between the p53 Arg72Pro polymorphism and endometriosis in Indian women; however, reduced expression of p53 was found in endometrial tissue in a small sample (n=5) of women with endometriosis.

Conclusions

The polymorphisms listed above represent only a small sample of those that have been investigated. No one single polymorphism has been consistently associated with endometriosis risk across multiple populations. For such studies to be of value, it is necessary that the normal and endometriosis patient population that is evaluated be of sufficiently large size, as some prior studies that have shown associations between a disease and a single nucleotide polymorphism that have been negated when a larger sample size was included. Ethnic variations also exist, which can confound data and prevent one from identifying a single global polymorphism linked to endometriosis risk.

Figure 8.1 The predicted 3-D structure for Estrogen Receptor-alpha (ER-α) Kumar, Raj, Mikhail N Zakharov, Shagufta H Khan, Rika Miki, Hyeran Jang, Gianluca Toraldo, Rajan Singh, Shalender Bhasin, and Ravi Jasuja. 2011. The dynamic structure of the estrogen receptor. *J Amino Acids* 2011: 812540.
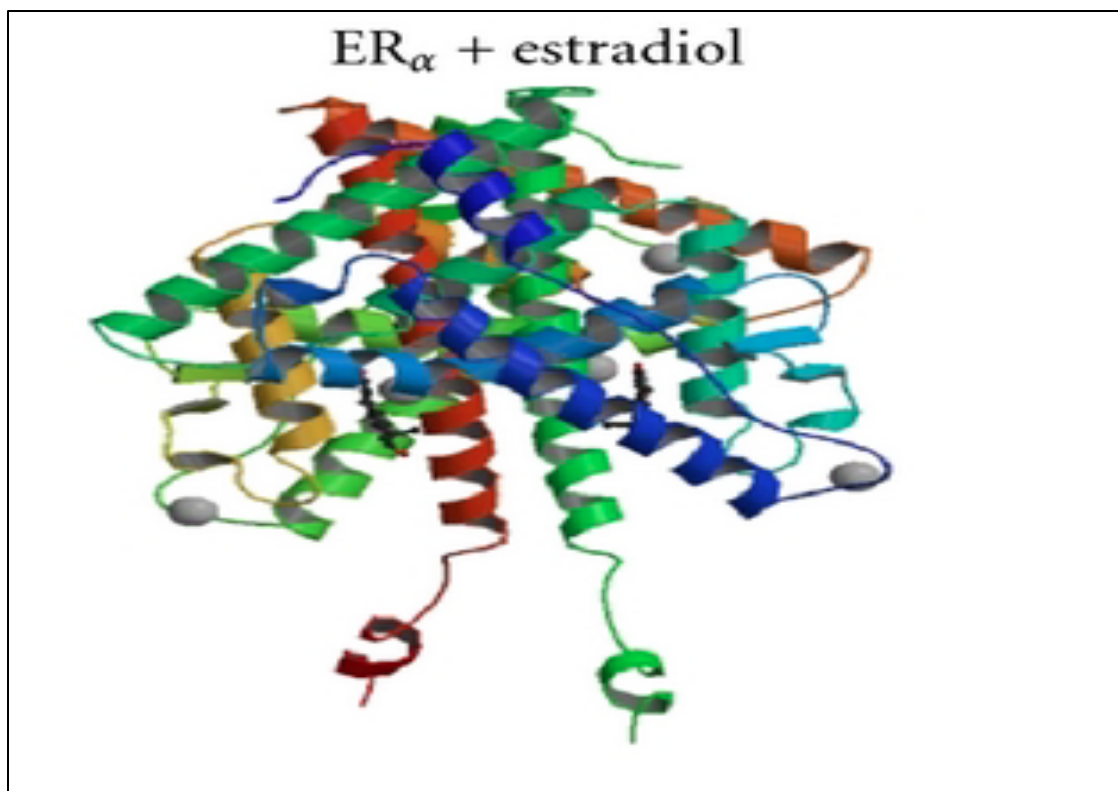
Figure 8.2. The predicted 3-D structure for Estrogen Receptor-beta (ER-β)
Kumar, Raj, Mikhail N Zakharov, Shagufta H Khan, Rika Miki, Hyeran Jang,
Gianluca Toraldo, Rajan Singh, Shalender Bhasin, and Ravi Jasuja. 2011. The
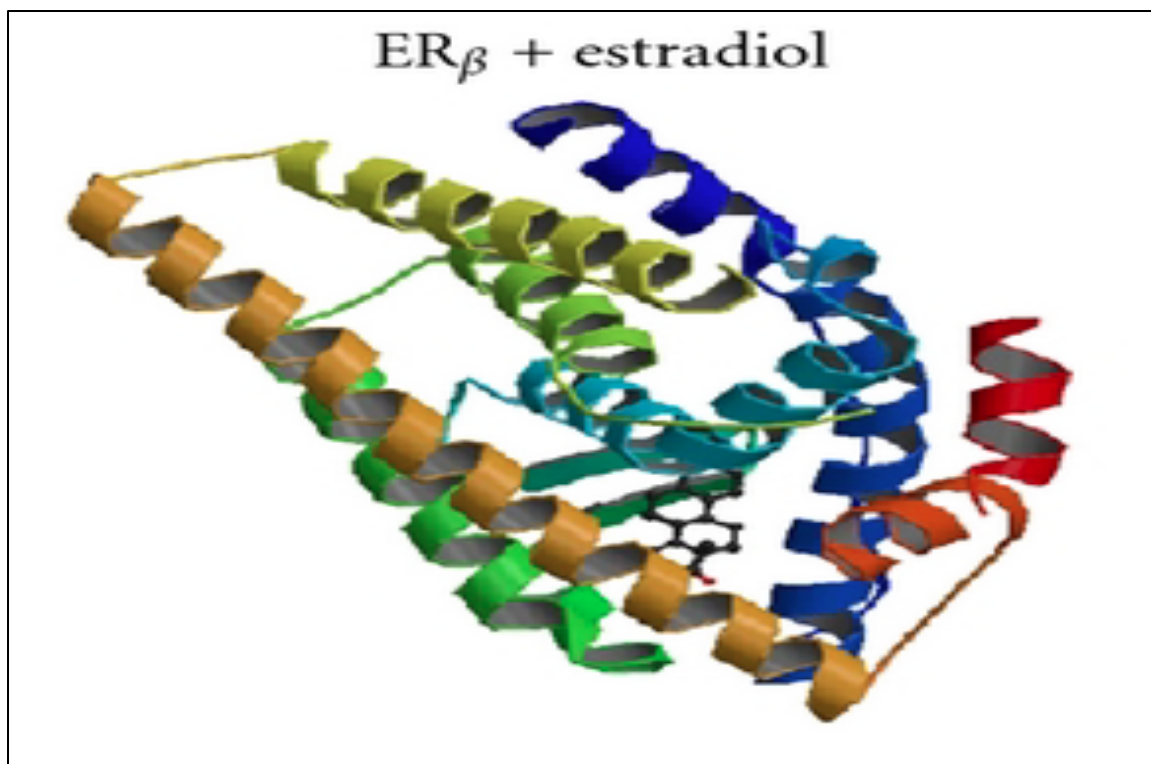dynamic structure of the estrogen receptor. *J Amino Acids* 2011: 812540.

Chapter IX: Animal Models

9.1 Overview of Animal Models

Due to the limited ability to perform experimental manipulations in humans, animal models have been developed in order to study the etiology of endometriosis. Humans and primates are the only mammals that develop spontaneous endometriosis and experiments involving primate models are incredibly expensive, which is why most studies involving primates have a small sample size. Small animal models using rodents that simulate this disease in humans have been developed in order to solve this dilemma; however, the differences between the human menstrual cycle and rodent estrous cycle present another problem with these studies. While there are limitations to studying this disease in the current animal models, such models have been useful for studying in vitro human samples, fetal development, and in vivo effects. This chapter will discuss the strengths, weaknesses, and findings of these endometriosis animal models.

9.2 Laboratory Mouse Model

Apparent differences exist between human and murine reproductive processes, which are necessary to understand when studying the endometriosis models involving the common laboratory mouse. Instead of a menstrual cycle,

female mice have an estrous cycle that lasts approximately four to five days. In order to identify the stage of the estrous cycle in a mouse, it is easy to perform a vaginal swab and determine the predominance of various vaginal cell types present at any given time (Caligioni 2009).

The 4-5 day estrous cycle can be divided into four stages: proestrus, estrus, metestrus, and diestrus respectively. The first stage of the cycle, proestrus, is characterized by a higher proportion of nucleated epithelial cells and occasionally keratinized cells in vaginal swabs. During proestrus, there is a thin layer of stratified squamous epithelium that begins to thicken and some of the epithelial cells undergo keratinization. This stage is the pre-ovulatory day when E2 increases, which results in the LH and FSH surge at night, and then ovulation occurs (Caligioni 2009). Estrus, the second stage of the estrous cycle, is identified by clusters of irregular shaped keratinized squamous epithelial cells with no visible nuclei. During estrus the stratified squamous epithelium has thickened and become fully keratinized. The superficial vaginal epithelial cells begin to disassociate and move into the lumen. The cytoplasm is observed to be granular and E2 remains elevated during the morning hours, but falls back to basal levels by the afternoon.

Metestrus is characterized by low plasma E2 levels and a mix of vaginal cell types, a majority of them being leukocytes, a few nucleated epithelial and keratinized squamous epithelial cells. During metestrus regression of the epithelium also occurs. The leukocytes within the stroma infiltrate the epithelium. E2 levels begin to rise again in the final stage of the estrous cycle, diestrus, and the highest percentage of cells in a vaginal lavage smear will be leukocytes (Conti 2004). The epithelium has thinned and mucified during this

stage and many of the leukocytes in the stroma migrate into the lumen through the epithelium.

A considerable physiological difference between mice and humans is that mice do not menstruate and therefore do not develop spontaneous endometriosis. This is a limitation of the murine model for endometriosis, but due to the low cost, the ability to study an in vivo setting, and the capacity to create knockouts for various genes in mice, the murine model is necessary. However, endometriosis must be induced in mice either surgically by implanting human endometrial tissue or by injecting endometrial tissue into the peritoneal wall (Tirado González 2010). There are two types of murine endometriosis models, the homologous model and the heterologous model, classified by the way in which the disease is induced.

The homologous murine model of endometriosis is developed via either surgical transplantation or by peritoneal injection of endometrial tissue from another mouse of the same strain (Tirado González 2010). Similar endometrial models have been achieved in various rodent species such as mice, rats, and hamsters. The mice receiving the tissue develop endometrioic lesions on the peritoneal wall, on the intestines, and on the uterine surface. In these animal models, both the donor and recipient are ovariectomized in order to prevent hormonal feedback from the ovaries and are given estrogen in order to simulate the estrous cycle. In the donor mice the exogenous estrogen treatment allows for the growth and proliferation of viable endometrial tissue for translocation. However, the estrogen treatment in the recipient mice may influence the progression of the disease due to its dependence on estrogen. One of the main problems with this model besides the physiological differences in menstrual and

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estrous cycles is that the endometrial lesions that develop in the murine models are much smaller and hard to distinguish.

The heterologous model was created using the athymic nude mouse, which is immunocompromised and therefore can accept xenographs of human endometrial tissue (Zamah 1984). The nude mouse has a mutation that causes the failure of thymus development and lack of hair follicles (Abbas 2012). T cell maturation occurs in the thymus and therefore the nude mouse does not have a T cell response. In the murine heterozygous model for endometriosis human endometrial tissue is surgically implanted in the peritoneal wall in order to study the effects of this tissue. However, the use of this immunocompromised mouse to study endometriosis pathogenesis is lacking the immune response aspect that would normally be present and potentially involved in the disease development. This is a limitation of the heterologous model along with other previously mentioned restrictions of rodent models.

9.3 Primate Model

Nonhuman primates are the only animals that have a menstrual cycle similar to humans. Merrill et al (1968) demonstrated that baboons are able to develop spontaneous endometriosis similar to humans, which is why they have been used as an animal model. A second study by D'Hoogne et al (1996) further supported this claim by observing histologically proven spontaneous endometriosis in a larger group of baboons over a three-year period. This study consisted of 67 laparoscopies performed on 24 baboons with normal pelvic cavities. These surgical procedures were done in 3-month intervals over a 32-month period. However, a study by Harirchian et al (2012) showed that the

surgical incisions made during laparoscopies caused endometrial lesions to develop in their control group; therefore the endometriotic lesions observed in prior studies (Merrill 1968) may have been caused by the laparoscopies themselves and not have been a spontaneous occurrence.

The olive baboon, Papio Anubis, is the most commonly used primate model for endometriosis. It is an important model due to its reproductive anatomy and menstrual cycle similarity to that seen in women (Stevens 1997). Endometriosis can be induced in the baboon by endocervical canal resection (Dehoux 2011) or by injecting autologous endometrial tissue into the peritoneal cavity (Dhooghe 1997). Induction by endocervical canal resection requires the removal of part of the cervical canal. The canal is then cauterized in order to avert menstrual bleeding into the vagina, which simulates retrograde menstruation. The second method of induction involves removing endometrial tissue from the uterus of the baboon and then injecting it into the peritoneal cavity. Both methods of endometriosis induction have been shown to induce this disease for study. However, studies involving these animals are expensive and require years for preparation and procedure.

9.4 Importance and Conclusions

The use of non-human primate models of endometriosis provides an examination of potential factors involved in the development of this disease as well as dysfunction of the immune response. Due to the physiological reproductive similarities between non-human primates and humans a greater understanding of the human pathology of this disease can be discovered. Problems encountered with the baboon model of endometriosis are the high cost

and the time needed in order to develop a disease model, which is why rodent animal models are also necessary.

Even with the limitations of both non-human primate and rodent models for comparative studies of endometriosis pathology, a lot has been learned about the factors and physiological disruption involved in the development and progression of this disease.

<center>Chapter X: Endometriosis Treatments</center>

10.1 Overview

Many pre-existing drugs have been used to treat endometriosis, however, because the etiology of this disease remains unknown, there is no cure. Due to years of research, understanding the involvement of certain factors has provided a method in which to treat particular symptoms. An important distinction, which must be made is whether a patient is looking for treatment of pain or infertility, because the treatment used will be based on the symptoms (Hoffman 2011). In patients with mild pain, NSAIDs, combination oral contraceptives, and progestins are used as treatment options; for infertility intrauterine inseminations in combination with empiric clomiphere is used. Moderate to severe pain as well as infertility in endometriosis patients can be treated with the surgical excision, ablation, and lysis of adhesions and further followed by post-operative treatments, such GnRH agonists and aromatase inhibitors, in order to prevent recurrence. This chapter will examine treatment options for pain and infertility as well as review the current literature and experimental treatment options of endometriosis.

10.2 Non-Steroidal Anti-Inflammatory Drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) work by non-selectively inhibiting cyclooxygenase isoenzymes. These enzymes function in prostaglandin

synthesis, which is responsible for endometriosis-associated pain and inflammation (Hoffman 2011). Cyclooxygenase, also referred to as prostaglandin H_2 synthase, exists in mammals as two isozymes (COX-1 and COX-2). These enzymes have different functions, but very similar amino acid sequences. While COX-1 is responsible for synthesizing the prostaglandins that regulate the secretion of gastric mucus in the GI tract, COX-2 produces prostaglandins responsible for inflammation, pain, and fever (Lehninger 2008). Studies designed to test the effects of decreased prostaglandin synthesis on the symptoms of endometriosis by inhibiting COX-1, were associated with unpleasant side effects such as stomach irritation and other serious conditions. After discovery of the organic structures of COX-1 and COX-2 in the 1990s, NSAID compounds were created that were specific to COX-2 in order to prevent negative effects of inhibition of both.

Ota et al (2001) demonstrated that ectopic endometrial tissue expresses higher levels of COX-2 than eutopic endometrium. Efstathiou et al (2005) using an in vivo treatment of a murine endometriosis model, found that NSAIDs produces differential suppression of endometriotic lesion establishment and growth as compared to controls. Also, Laschke et al (2007) showed that treatment of a murine model of endometriosis with COX-2 inhibitor showed a 57% reduction in endometrial lesions compared to controls. In addition, COX-2 inhibitors decreased cell proliferation and VEGF levels, which ultimately resulted in smaller endometriotic lesions and suppression of angiogenesis. Although these animal models have shown disease regression with NSAID

treatment, few studies have critically evaluated the effectiveness of disease regression in women with surgically confirmed endometriosis (Hoffman 2011).

While there is good evidence for pain relief in women with dysmenorrhea and pelvic pain using COX-2 inhibitors, the long-term use of these drugs can cause cardiovascular problems, and should therefore be used in moderation and for as short a period of time as possible (Nasir 2004). For this reason, other methods of disease regression are also being studied.

10.3 Combination Oral Contraceptives

Combination oral contraceptives were introduced in the United States (US) in May 1960 as a method of family planning and pregnancy prevention (Hatcher 2007). The first oral contraceptive, Enovid, contained 150 μg of mestranol and 9.85 mg of norethynodrel. These high levels of hormones were used due to their effectiveness and because studies had not been performed to identify the lowest effective dose. Today, the concentration of steroid hormones found in combined oral contraceptives (COCs) has declined dramatically, which have made these drugs much safer and lessened their side effects. All COCs have an estrogen and progestin component, which interact with each other. Synthetic progestins have higher androgenic effects, which can counter some of the metabolic impact of estrogen on the body; while estrogen can also suppress some of the progestin effects. These hormone interactions are dependent upon the concentration of estrogen and progestin in a particular pill.

Two forms of estrogen are used in COCs in the United States, including mestranol and ethinyl estradiol (EE) (Hatcher 2007). Mestranol is a drug precursor, which must first be metabolized by the liver into EE before it becomes

biologically active and effects can take place. Fifty micrograms of mestranol is equivalent to 35-40 µg of EE, which is more potent and has a longer action time than the steroid estradiol normally produced by the body. In COCs prescribed in the US, EE varies from 20 µg to 50 µg per pill. Progestins, the second component of COCs, are found in eight different forms in the US. Natural progesterone is not very well absorbed by the GI tract, is readily metabolized, and high doses cause fatigue. For these reasons, COC developers began looking at long-lasting compounds (C-19 androgens) to use as the progestin component of the pill rather than natural forms.

The eight different progestins have made up various COCs and exist under different "progestin generations" that have developed over time (Hatcher 2007). The first generation of progestins includes norethindrone, norethindrone acetate, and ethynodiol diacetate. When low dose amounts of progestin are used, typically norethindrone will be included. The main side effects of the first generation progestins was spotting and untimely bleeding. In an attempt to control the side effects of the first generation, the second generation of progestins, was created. These progestins (norgestrel and levonorgestrel) are more potent, have incredibly long half-lives, and have more androgenic activity than the first generation of progestins. A third generation of progestins (desogestrel and norgestimate) was created to exert a lower androgenic activity than the second generation and to main an increased progestational activity. The decreased androgenic activity of these third generation progestins allowed for a fuller metabolic effect of the COCs estrogen component.

There are two formulations of COCs available: monophasic and multiphasic. Monophasic contraceptive indicates that each active pill is composed of the same doses of the estrogen and progestin as the other active pills; whereas multiphasic signifies that the amounts of hormones in the active pills vary (Hatcher 2007). Traditionally, monophasic COCs are prescribed for the treatment of endometriosis, but no studies offer proof in support of their clinical superiority to multiphasic COCs (Hoffman 2011).

The mechanism of action of COCs includes reducing menstrual flow and potentially the volume of retrograde menses, decreasing GnRH pulses and pituitary responsiveness to GnRH stimulation, suppression of LH and FSH synthesis, and in some cases suppressing the mid-cycle LH surge (Maia 2008). By suppressing the hormones required for ovulation, ovarian estradiol production decreases, removing the stimulus for endometriotic implants and reducing the risk of developing endometriotic lesions. COCs also work by causing the pseudo-decidualization of these endometriotic lesions. Pseudo-decidualization is caused by progestin induction of terminal differentiation of endometrial cells, which stops their growth. The progestin component of COCs, therefore results in reduced symptoms of endometriosis during use. However, this treatment does not cure the disease, because lesions only stop growth during treatment and are ready for re-growth when COC use is discontinued (Hatcher 2007).

10.4 Surgical Treatment of Endometriosis

The principle manner in which endometriosis diagnosis is confirmed is via laparoscopy; for this reason, surgical treatment of the disease at the time of diagnosis seems appropriate (Hoffman 2011). Surgery can be used to treat either

symptom of endometriosis—moderate to severe pain or infertility. The benefits of surgery for individuals with severe forms of this disease include a mended pelvic anatomy, excision of implanted endometriotic lesions, and ultimately decreased inflammation (Macer 2012). There are two methods of endometriotic lesion removal: excision or ablation. Excision involves the complete removal of the lesion and ablation entails the destruction of ectopic endometrial tissue with conventional electrosurgery or a high-energy laser.

According to a study by Blackwell et al (1991) laser ablation does not prove to be more effective than electrosurgery. However, regarding laparoscopic ablation versus the excision of endometriomas, excision was associated with a subsequent increased spontaneous pregnancy rate in women who were documented as infertile. A randomized controlled study consisting of 341 women was conducted in order to discover whether laparoscopic surgery increased the fertility rate of endometriosis-associated infertility. It was concluded that ablation of endometriotic lesions significantly enhanced the fertility of women compared with diagnostic laparoscopy alone (Macer 2012).

Vercellini et al (2013) compared surgical treatment of endometriosis (ablation via electrosurgery) to a low-dose progestin treatment for disease-associated deep dyspareunia in 192 women diagnosed with stage III and IV endometriosis. This study compared the Female Sexual Function Index (FSFI), a 19-item questionnaire that evaluates the major groups of female sexual dysfunction and sexual satisfaction, of individuals undergoing surgery to those taking a progestin treatment. Patients that received surgery observed a substantial and rapid benefit (increased FSFI score) compared to the progestin treatment group. However, over the course of the study, performance of the

surgery group on the FSFI deteriorated and no significant difference was observed between the two groups in the long term.

These studies indicate an initial suppression of symptoms after ectopic endometrial implants are removed, but suggest a recurrence of symptoms potentially due to regrowth of endometriotic lesions months after the initial excision. Also, the surgery itself can potentially cause other lesions to grow (Harirchian 2012), which also may be responsible for the return of endometriosis-associated pain. Typically post-operative treatments for both pain and infertility are necessary for successful liberation from symptoms. However, even with a combination of surgery and post-operative treatments, a percentage of patients have a recurrence of symptoms.

10.5 Post-operative Treatments

In two studies of 95 participants a statistically significant reduction in the recurrence of dysmenorrhea was observed in a group that received post-operative treatment than in the control group that only had surgery (AbouSetta 2013). The post-operative treatment in this review was a levonorgestrel-releasing intrauterine device (IUD). As previously mentioned levonorgestrel is a potent progestin that decreases the metabolic activity of estrogen. This treatment suppresses the effects of estrogen and has a similar mechanism of action to the other post-operative treatments that are used.

GnRH causes the release of the gonadotropin hormones and subsequent ovarian steroidogenesis and ovulation. However, continuous, nonpulsatile GnRH administration causes the anterior pituitary to become desensitized and decrease ovarian steroidogenesis (Hoffman 2011). This result allows GnRH

agonists to be used to treat endometriosis. Ultimately, GnRH agonists cause loss of estradiol production, which is necessary for the implantation and development of endometriotic lesions. This post-operative treatment allows for a pseudo-menopausal state and allows the body to recover, while simultaneously suppressing the disease from reforming.

The third class of post-operative treatments, aromatase inhibitors, works by a mechanism similar to that of its counterparts. As previously mentioned aromatase is an enzyme that catalyzes the conversion of testosterone to estrogen; therefore aromatase inhibitors would decrease the amount of estrogen produced by the ovaries. In one study, aromatase inhibitors were used for the treatment of severe menopausal endometriosis (Takayama 1998). This treatment was given after a woman underwent a complete hysterectomy and a bilateral salpingo-oophorectomy, in which both of the woman's ovaries and fallopian tubes were removed. This individual experienced significant pain relief and reduction of lesion size after nine months. A second study, involving a baboon model for endometriosis, showed a significant reduction in endometrial ectopic lesion size when treated with aromatase inhibitors, whereas the control group given a placebo experienced endometrial lesion growth (Langoi 2012). These post-operative treatments have shown to be necessary in successful suppression of this disease. These studies reconfirm the role of estrogen in endometriosis sustenance.

Chapter XI: Conclusion

Many factors play a role in the development and pathology of endometriosis, which explains why the etiology of this disease remains elusive. Current research supports the theory of retrograde menstruation along with the dysfunction of the immune response to misplaced endometrial tissue and increased secretion of angiogenic factors by ectopic endometrium. Other such factors that potentially increase endometriosis-associated inflammatory responses are environmental toxicants and inherited genetic variation, otherwise known as single-nucleotide polymorphisms, which alter various genes that are potentially involved in endometriosis pathogenesis.

The only method to absolutely diagnose endometriosis is by surgical laparoscopy. Because it has been discovered that surgery alone can induce endometrial lesion formation, alternative modes of diagnosing endometriosis, such as by ultrasound, should be a developed. The current classification system of endometriosis by the American Fertility Society does not provide information on the severity of pain or fecundity. The Endometriosis Fertility Index attempts to bridge this gap, however, it is not currently a widely accepted method of identifying fertility in endometriosis patients. In order for preventative measures to be taken an endometriosis-associated pain predictor is needed.

Animal models, even with their limitations, have brought us steps closer to understanding steroid responses, angiogenic and immunologic factors

produced in vivo, and the role of certain genes using knock-out mice. However, a higher level of understanding may not be obtained without more clinical trials. Treatments exist to aid in suppressing the symptoms of endometriosis, but due to the elusive nature of the disease, there is no cure. Endometriosis is a complex, multi-factorial disease; and while we've gained valuable knowledge on the potential factors involved, further research is necessary to achieve a better understanding of the pathogenesis of this disease.

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