Deep infiltrating endometriosis environment, genetics, epigenetics

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DEEP INFILTRATING ENDOMETRIOSIS

ENVIRONMENT, GENETICS, EPIGENETICS

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DEEP INFILTRATING ENDOMETRIOSIS

ENVIRONMENT, GENETICS, EPIGENETICS

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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Stel dat je niet naar huis gaat
Je laat de laatste ronde gaan
Je planned alleen de eerste meters
De hardste hier vandaan

Stel dat je hoofd je hart volgt
En je loopt naar het station
En je leunt op de gedachte
Terwijl je wacht op het perron

Het maakt niet uit wat je gisteren deed
Het gaat om dat wat je morgen pas weet

Acda en de Munnik

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Abbreviations

ASMA	alpha-smooth muscle actin
bp	base pair
cDNA	complementary DNA
CI	confidence interval
CT	comparative threshold
DNMT	DNA methyltransferase
DZ	dizygotic
ECM	extracellular matrix
ESC	embryonic stem cell
E1	estrone
E2	estradiol
FM	fibromuscular
HDAC	histone deacetylase
he	heterozygous
hmC	hydroxymethylation of cytosines
ho	homozygous
HSD	hydroxysteroid dehydrogenase
HWE	Hardy-Weinberg equilibrium
IBD	identity by descent
Ig	immunoglobulin
IGFBP	insulin growth factor binding protein
IL	interleukin
IVD	in vitro methylated DNA
LFC	Leuven Fertility Center
LRES	long-range epigenetic silencing
MBD	methyl-CpG-binding domain protein
miRNA	micro-RNA
mRNA	messenger RNA
MSP	methylation specific PCR
MUMC	Maastricht University Medical Center
MZ	monozygotic
NL	normal lymphocytes
OR	odds ratio
P	progesterone
pAbs	polyclonal antibodies
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PR	progesterone receptor
QTL	quantitative linkage analysis

rAFS	revised American Society for Reproductive Medicine
RFLP	restriction fragment length polymorphism
RT-PCR	real-time PCR
TGF- β	transforming growth factor- β
T β R	transforming growth factor- β receptor
SD	standard deviation
SEM	standard error of the mean
SI	staining index
SM	smooth muscle
SMM	smooth muscle metaplasia
SM-MHC	smooth muscle myosin heavy chain
SNP	single nucleotide polymorphism
TSS	transcription start site
T1	wildtype allele
T2	mutant allele
wt	wildtype

Chapter 1

General Introduction

Endometriosis

Clinical Introduction

Endometriosis is defined as the presence of functional endometrial glands and stroma at ectopic locations outside the uterine cavity. Ectopic endometrial implants are most often found on the ovaries, the fossa ovarica, the uterosacral ligaments and the posterior cul-de-sac. A proportion of women with this condition may be asymptomatic, but symptoms can be substantial and include dysmenorrhea, dyspareunia, chronic and severe pelvic pain throughout the menstrual cycle, and infertility. Endometriosis predominantly develops in women of reproductive age and regresses after menopause or after ovariectomy (1), suggesting that the establishment and growth of ectopic implants is dependent on ovarian steroids, similar to the situation encountered in eutopic endometrium. Being one of the most common benign gynecological conditions with an estimated prevalence of about 10% (2), endometriosis is a debilitating disease with detrimental effects on social, occupational and psychological functioning (3). The prevalence increases up to 30% in patients with infertility and up to 45% in patients with chronic pelvic pain (2,4). At present, endometriosis can only be reliably diagnosed by laparoscopy, with subsequent biopsy of suspect lesions for histological confirmation. Because the symptomatology of endometriosis shows numerous commonalities with a wide range of diseases, a diagnostic delay of 5 to 10 years is not uncommon in some healthcare settings (3).

Pathogenesis

Although endometriosis stands as one of the most investigated disorders of gynecology, our current understanding of the pathogenesis of this disease remains limited. Theories that have been developed regarding this subject can be divided into three main concepts: 1) the *in situ* development theory, i.e. the concept that endometriosis develops *in situ* from local tissues, such as the germinal epithelium of the ovary, remnants of the Wolffian or Müllerian ducts or from metaplasia of totipotent mesothelial serosal cells, 2) the induction theory, i.e. the concept that endogenous substances released from degenerating menstrual endometrium induce a metaplastic process in undifferentiated mesenchyme to develop into endometrial tissue (5) and 3) the retrograde transplantation theory, which is currently the most widely accepted theory regarding the pathogenesis of endometriosis (6). According to this theory, reflux of viable endometrial cells takes place through the Fallopian tubes during menstruation, with subsequent adhesion, implantation and growth on and into the peritoneum and ovary. A large body of experimental and clinical observations add support to the Sampson hypothesis, such as the anatomical distribution of endometriosis lesions in the abdominal cavity (7), the demonstration of viable endometrial cells in the peritoneal fluid of women with endometriosis (8, 9) and characteristics of retrogradely shed endometrial fragments such as the ability to adhere to

the peritoneal lining (10), to invade the extracellular matrix (11, 12) and to produce angiogenic factors that can potentially induce local neovascularisation (13). However, research indicates that menstrual reflux is a common occurrence, and small endometriotic implants occur frequently in asymptomatic women of reproductive age (14, 15). This has led to the hypothesis that mild peritoneal endometriosis is a condition that occurs intermittently in most if not all menstruating women with patent tubes (16). As a consequence, there is a growing appreciation for the notion that mild peritoneal endometriosis should be considered a physiological phenomenon rather than a disease.

It has been proposed that viable endometrial fragments arriving in the peritoneal cavity through retrograde menstruation would normally be cleared by a cell-mediated immune response. The incapacity to clear the peritoneal cavity of endometrial cells may either result from innate properties of the endometrium itself, leading to increased survival, or from (immunological) aberrations in the local peritoneal environment, leading to decreased surveillance, recognition and destruction of the misplaced endometrial cells. When these local pelvic clearance mechanisms fail, endometriosis lesions may have the opportunity to adhere, vascularize, grow, and infiltrate, thus allowing the “phenomenon” endometriosis to progress into the “disease” endometriosis. Endometriotic disease was characterized by Koninckx and coworkers as the presence of either 1) considerable pelvic adhesions perturbing the local anatomical situation, 2) endometriotic ovarian cysts or 3) deep infiltrating endometriosis lesions (16). The question that has frustrated investigators for more than a century is: why does endometriosis develop into a pathological condition in some but not all women?

Deep infiltrating endometriosis

Definition and pathogenesis

In the early nineties of the past century, deep infiltrating endometriosis was introduced as a new distinct entity (17). Deep infiltrating endometriosis is defined as endometrial glands and stroma infiltrating >5 mm under the peritoneal surface. The threshold of 5 mm was chosen because of several reasons. First, lesions infiltrating >5 mm were shown to be the most active lesions from a morphological point of view (17). A second argument is derived from the frequency distribution of the depth of infiltration in women with pelvic pain and/or infertility, which shows a biphasic pattern with a nadir at 5-6 mm (18). Furthermore, of all lesion types, deep infiltrating endometriosis showed the strongest association with pelvic pain. Women with pain have larger and deeper lesions and very deep implants (>10 mm) were found exclusively in patients with pain. Therefore, it was suggested that in most women, peritoneal endometriosis lesions infiltrate only superficially, cause little or no symptoms and are eventually inactivated. Lesions that infiltrate deeper than 5 mm become a different condition. The disease becomes more

active and aggressive, and develops into even deeper lesions causing pelvic distortion and severe pain. Deep infiltrating lesions are often symptomatic and are strongly associated with symptoms such as dysmenorrhea, deep dyspareunia, chronic pelvic pain and painful defecation (19) .

In recent years, the pathogenesis of deep endometriosis has also been the subject of another debate. It has been argued that deep infiltrating endometriosis may be a dissimilar disease entity, different from peritoneal and ovarian endometriosis. Several arguments exist supporting this notion. Deep infiltrating endometriosis lesions have a distinct histological appearance resembling adenomyosis (i.e. the presence of endometrial glands and stroma in the myometrium) of the uterine wall. They are nodular in appearance and mainly consist of fibromuscular tissue, interspersed with islands of endometrial glands and stroma (20). Moreover, deep infiltrating endometriosis lesions have a specific anatomical distribution, as they are mainly found in the pouch of Douglas and on the uterosacral ligaments. Therefore, it was hypothesized by some that this type of lesion is not caused by implantation of regurgitated endometrial tissue during menstruation (i.e. Sampson's theory), but rather develops from metaplasia of Müllerian remnants located in the rectovaginal septum, in line with the *in situ* metaplasia theory (21). Alternatively, there are those that believe that deep infiltrating endometriosis lesions develop from superficial peritoneal lesions in the pouch of Douglas (22).

Fibrosis and smooth muscle metaplasia in deep infiltrating endometriosis

Although the question whether deep endometriosis and adenomyosis represent the same disease entity is still a subject of debate, a certain histological distinction can be made between deep infiltrating endometriosis on the one hand, and peritoneal and ovarian endometriosis on the other hand. Deep endometriosis lesions are nodular in appearance and are histologically characterized by islands of endometrial glands and scanty stroma interlacing dense tissue composed of fibrous and smooth muscle cells, whereas peritoneal and ovarian endometriosis are characterized by a more glandular appearance surrounded by a cytogenic stroma. Strikingly, the major component of the deep infiltrating nodular lesion is not endometrial tissue but fibromuscular tissue (20, 21, 23). Itoga and coworkers demonstrated that fibrosis was present in 89/90 histological specimens of rectovaginal endometriotic nodules. Moreover, there was a significant correlation between the amount of fibrosis and the amount of endometrial tissue present in the lesion. Aggregated smooth muscles that were not associated with blood vessels, defined as smooth muscle metaplasia (SMM), were always present in fibrotic areas, and the degree of SMM in the entire tissue was significantly correlated with the degree of fibrosis. These authors were not the first to histologically characterize endometriosis lesions: in 2000 Anaf and coworkers demonstrated that smooth muscles are present in peritoneal, ovarian, uterosacral and rectovaginal lesions. Smooth muscle cells are absent in the unaffected peritoneum and in the eutopic endometrium of women with and without pelvic

endometriosis (23), suggesting a role of the local environment in the development of these fibromuscular cells. As a result, it was speculated that the smooth muscle component in endometriosis lesions either results from the capacity of the totipotent coelomic serosal cells to differentiate not only into endometrial glands and stroma but also into smooth muscle, or from the fibromuscular differentiation of regurgitated endometrial stromal cells.

Transforming growth factor-beta signaling and fibrosis

Among the many factors that modulate the formation of fibrosis, transforming growth factor-beta (TGF- β), and especially the isoform TGF- β 1, is the cytokine most causatively associated with disorders characterized by fibrosis throughout the body (24). The TGF- β isoforms TGF- β 1, TGF- β 2 and TGF- β 3 are pleiotropic cytokines that mediate a variety of effects on a range of cell types. After activation, the TGF- β s bind with high affinity to TGF- β receptor (T β R) II which phosphorylates T β RI, leading to the recruitment and phosphorylation of the intracellular downstream effector proteins Smad2 and Smad3. Phosphorylated Smad2 and Smad3 subsequently bind to Smad4 and translocate to the nucleus to initiate target gene expression. TGF- β 1 exerts its fibrogenic effects by promoting expression of extracellular matrix (ECM) genes and suppressing the activity of enzymes that degrade ECM (such as matrix metalloproteinases). Furthermore, TGF- β enhances fibroblast proliferation, and induces their differentiation into the myofibroblast phenotype.

TGF- β 1 has been implicated in both the physiologic growth and differentiation of the endometrium as well as in the pathogenesis of endometriosis. All TGF- β s and their high affinity receptors are stage specifically expressed in both endometrial glands and stroma (25), suggesting that their expression is under hormonal control. Since mRNA and protein expression of all three TGF- β s is increased around menstruation, it was suggested that TGF- β s might be involved in initiation of menstruation. TGF- β 3 is thought to participate in the postmenstrual regeneration of endometrium, because of high expression levels in this phase of the menstrual cycle (26). Higher concentrations of TGF- β 1 have been measured in the peritoneal fluid of endometriosis patients compared to controls (26-28), suggesting that this cytokine may be crucial in the establishment and/or maintenance of endometriosis.

Progesterone resistance

Evidence for progesterone resistance in endometriosis

Endometriosis is an estrogen dependent disease, demonstrated by the fact that it occurs primarily in cycling women of reproductive age (2, 6) and regresses after menopause or

after ovariectomy. Progesterone is the most potent antagonist of estrogen-induced proliferation in the endometrium and as such may play a pivotal role in the pathogenesis of endometriosis. Progesterone induces differentiation of both endometrial stromal and epithelial cells, exemplified at the histological level by pseudodecidual and secretory changes, and reduces mitotic figures and cell proliferation. Another striking effect of progesterone on the endometrium is to stimulate the expression of the enzyme 17 β -hydroxysteroid dehydrogenase type 2 (17 β -HSD type 2) in epithelial cells. 17 β -HSD type 2 catalyzes the conversion of the biologically active E2 into the biologically less active estrone (E1) (29). In women with endometriosis, this enzyme is expressed in eutopic endometrium in the luteal phase of the menstrual cycle, but not in ectopic endometriotic lesions simultaneously biopsied from the same patient (30). The lack of 17 β -HSD type 2 in ectopic endometriotic tissue during the luteal phase, despite histologically recognizable secretory changes, is suggestive of selective resistance to progesterone action. Eutopic endometrium of endometriosis patients also exhibits signs of progesterone resistance since a number of progesterone target genes, such as glycodeilin, was shown to be deregulated in eutopic endometrium of endometriosis patients during the window of implantation, at which time the endometrium is exposed to the highest levels of progesterone (31). Finally, failure of endometriosis to regress in response to treatment with progestins in a significant number of patients is another, clinical indicator of progesterone resistance (32, 33).

Progesterone receptor

The effects of progesterone are mediated through intracellular progesterone receptors that are expressed from a single gene as two protein isoforms, progesterone receptor A (PR-A) and progesterone receptor B (PR-B) (34). The PR-A and PR-B isoforms are ligand-dependent members of the nuclear receptor family that are structurally identical, except for an additional 164 amino acids at the amino terminal of PR-B (35). This region encodes a transactivation function that is specific to PR-B and is required to specify target genes that can be activated by PR-B but not PR-A (36). PR-B functions as a stronger transcriptional activator of progesterone target genes, whereas PR-A has been shown to act as a dominant repressor of PR-B (37, 38). Furthermore, the responses of ligand-activated PR-A and PR-B strongly depend on the cellular context. The physiologic importance of maintaining correct relative expression levels of PR isoforms in tissues is underlined by the fact that loss of coordinate expression of PR isoforms is an early event in breast carcinogenesis (39). Alteration of the PR-A/PR-B ratio in breast and endometrial carcinoma cells has been shown to favor cellular invasion and metastasis (40, 41). Moreover, high PR-B levels often are found in highly malignant endometrial cancers, and selective ablation of the A isoform in knockout mice results in endometrial hyperproliferative and premalignant changes. Hence, the anti-proliferative effects of progesterone are thought to depend on a tight regulation of the PR-A/ PR-B isoform

balance. Several studies have reported aberrant expression of progesterone receptor in eutopic and ectopic endometrium of endometriosis patients. For example, both PR-A and PR-B were present in eutopic endometrium of endometriosis patients, but PR-B was completely absent in ectopic endometriotic lesions (42). Wu and coworkers also demonstrated significantly reduced expression of PR-B in epithelial cells of endometriotic lesions, associated with hypermethylation of the PR-B promoter region (43). Furthermore, a lower PR-B/PR-A ratio in eutopic endometrium of endometriosis patients compared to disease-free controls was demonstrated by western blotting (44). Hence, the reported disruption of PR-A/PR-B ratio in endometriotic tissue might lead to an impaired stromal differentiation and a consequent relative resistance to P action. Factors influencing the relative expression levels of PR-A to PR-B may therefore be of importance in the pathogenesis of endometriosis.

Genetics

Genetic contributions to endometriosis

Endometriosis is commonly regarded as a complex trait, caused by the interplay between genetic and environmental factors. Evidence for the contribution of genetic factors to the individual susceptibility for endometriosis has been provided by numerous studies (45-52), demonstrating familial clustering and increased prevalence among first degree relatives and in twins. A strong familial tendency has also been reported in non-human primates, further supporting a genetic predisposition to the disorder (53).

Higher rates of endometriosis are found among the relatives of endometriosis cases compared with those of both hospital (50, 54) and population-based (51) controls. In an Australian sample of twins and their families, the relative recurrence risk to sibs has been estimated at 2.34 (52). However, estimates from imaging studies on the sisters of women with more severe disease suggest that the relative risk may be as high as 15 (55). Overall, the risk to first-degree relatives of patients for developing endometriosis has been reported to be 5-8%.

Stefansson and coworkers estimated the contribution of genetic factors in endometriosis in the Icelandic population, which is known to have limited genetic variability because of its isolated geographical position (51). These authors demonstrated that 750 women with endometriosis were significantly more interrelated than matched control groups. The risk ratio for sisters was 5.20 and for cousins 1.56. The average kinship coefficient for endometriosis patients was significantly higher than that calculated for 1000 sets of 750 matched controls, which is consistent with a complex genetic basis of this disease.

Another type of study design that is suitable for studying the impact of genetic factors is the twin study, which compares the concordance rate (the percentage of twin pairs who both have the disease) between monozygotic (MZ) and dizygotic (DZ) twins. Increased concordance in MZ compared with DZ twins suggests a role for genetic factors in disease

causation, assuming that MZ twins do not share more environmental influences than DZ twins. In the case of endometriosis, higher concordance for MZ than DZ twins has consistently been observed (54, 56, 57). A large-scale study in 3096 twins on the Australian National Health and Medical Research Council Twin Register showed a 2.0% concordance for monozygotic and 0.6% for dizygotic twins (58). Moreover, these authors concluded that 51% of the variation in endometriosis risk may be attributable to genetic factors.

Mode of inheritance

The 5-8% risk observed for first-degree relatives is more consistent with a polygenic/multifactorial mode of inheritance than with a single mutant gene (Mendelian or monogenic mode of inheritance). However, the risk of recurrence is slightly higher than expected for polygenic inheritance (generally 2-5%), indicating that one or more Mendelian forms could coexist. Still, a polygenic mode of inheritance is more likely if endometriosis is assumed to be a single disease entity. The polygenic model postulates that a given disease is caused by cumulative individual effects of many different genes acting together in an additive fashion. If an individual carries a relatively large number of these genes, there is a threshold beyond which the disease is manifested.

According to the polygenic/multifactorial model, first-degree relatives will have more susceptibility genes and will be more frequently affected than the general population, which is indeed the case in endometriosis. Additional support for polygenic inheritance is the increased severity in familial cases. According to the polygenic model, the severity of a polygenic disorder increases with increasing underlying genetic liability. Hence, the higher the proportion of affected relatives, the greater the likelihood that the proband has severe endometriosis. The fact that there is an earlier age of onset for familial versus nonfamilial cases and a similar age of onset among affected relatives also supports a polygenic mode of inheritance (59).

Strategies for identifying causative genes

Finding genetic variants that contribute to complex diseases such as endometriosis is a difficult task because the contribution of individual genes is small, many genes contribute to an individual's risk of developing the disease and disease risk is often modified by other, environmental factors. However, common diseases present a much greater burden to public health than Mendelian diseases, that are generally much rarer. Therefore, considerable (international) efforts are made to define genetic contributions to these diseases. The most widely used general approaches so far in the search for genes causatively related to the pathogenesis of endometriosis are genome-wide quantitative linkage analysis (QTL), microarray technology, and the candidate gene approach based on biological plausibility. An overview of these techniques and a summary of their main advantages and disadvantages is presented in Table 1.

In the past decades, many genes that were implicated in monogenic (Mendelian) diseases have been identified by QTL. Although this method has been successful in identifying high relative risk genes, it has not been successful in identifying genes that are involved in complex traits. This can be explained by several key features of complex disease. First, complex diseases typically vary in severity of symptoms and age of onset, which makes it difficult to define an appropriate phenotype and select the best population to study. Second, complex diseases may vary in their etiological mechanisms, which may involve various biological pathways. Third, and probably most important, complex diseases are more likely to be caused by several (and even numerous) genes, each with an overall small contribution and relative risk.

The candidate gene approach focuses on genes that are selected because of *a priori* hypotheses about their etiological role in disease, rather than rely on markers that are evenly spaced throughout the genome without regard to their function or context. In the case of endometriosis, endometriotic tissue may have an increased capability to adhere, proliferate, implant and survive in ectopic locations compared to normal endometrium. Genes that are known to be involved in the regulation of these processes are often selected as candidate genes for conducting genetic association studies. This is based on the premises that the susceptibility for endometriosis is caused by variations in DNA sequence (i.e. single nucleotide polymorphisms) in the selected candidate gene. In this respect, the preferred strategy is to search for SNPs with known functional consequences, meaning that they either change gene expression or the structure of the protein for which it codes, because these are most likely to affect the risk of a phenotype. Genes and their polymorphic variants that have been investigated so far with respect to the possible association with endometriosis mainly include genes involved in inflammation (cytokines, chemokines and their receptors, growth factors, Human Leukocyte Antigens, nitric oxide, adhesion molecules), steroid hormone regulation, metabolism and biosynthesis, detoxification, tissue remodeling, vascular function, and cell-cycle regulation.

As described earlier, both the formation of fibrosis and progesterone resistance are presumed etiological mechanisms in the pathogenesis of deep infiltrating endometriosis. TGF-beta 1 and progesterone receptor are pivotal genes involved in these respective processes, rendering both these genes plausible candidates for genetic association studies. Overproduction of TGF beta 1 is implicated in the pathogenesis of several fibrotic diseases at various sites throughout the body. Deep infiltrating endometriosis lesions are largely composed of fibromuscular tissue, and mechanisms increasing the fraction of biologically active TGF beta 1, such as single nucleotide polymorphisms, may increase the susceptibility for developing this type of endometriosis. The -509C/T polymorphism is located in the promoter region of the TGF beta 1 gene and is associated with a significant increase in TGF-beta 1 plasma concentration, to the extent that 10% of the genetic variance in TGF beta 1 plasma concentration can be attributed to the presence of this polymorphism (60).

Table 1 Strategies used for the identification of causative genes involved in endometriosis.

Strategy	QTL	Microarray technology	Candidate gene approach
Principle	Requires no information concerning candidate genes or chromosomal loci (non-hypothesis driven)	Requires no information concerning candidate genes or chromosomal loci (non-hypothesis driven)	Selection of genes based on biological plausibility, requires knowledge of biological pathways involved in disease (hypothesis driven)
Advantages	Pivotal genes can be identified if the same chromosomal locus shows allele sharing in different families of affected individuals (Identity by descent; IBD)	Differentially expressed genes may point to causative association with the disease	Susceptibility for endometriosis is caused by a variation in DNA sequence that alters: -gene expression or -structure of protein for which it codes
	Can be detected irrespective of (incomplete) penetrance, phenocopy, or genetic heterogeneity	Many potential relevant genes can be tested simultaneously	Genes do not have to be expressed at the time that tissue is obtained
Disadvantages	Need for multigenerational families of affected and unaffected individuals	Need for an invasive procedure (laparoscopy, pipelle biopsy, hysteroscopy) to obtain endometrial tissues	Genetic associations often inconsistent across ethnic barriers
	Need for an invasive procedure (laparoscopy) to reliably confirm the absence of endometriosis in presumably unaffected individuals	True causative gene may not be expressed at the point in time when the tissue was studied	Insufficient statistical power to detect variants with expected small effect size (small sample sizes)
	Possible interaction between different chromosomal loci	Genes expressed may have little to do with causality or disease progression	Publication bias: negative associations less likely to be published

As demonstrated in progesterone deficient mice, the physiological effects of progesterone depend completely on the presence of the human progesterone receptor (61). Within this context, structural changes in the PR and factors influencing the relative expression levels of PR-A to PR-B may be of importance in the pathogenesis of endometriosis. To date, several polymorphisms with (possible) functional consequences in the PR gene have been identified. The PROGINS polymorphism consists of a 320 bp PV/HS-1 Alu insertion in intron G, and two point mutations, a silent point mutation in exon 5 (H770H) and a single amino acid change in exon 4 (V660L). The functional consequences of the PROGINS polymorphism are not yet fully understood, but there are indications that the PROGINS variant of PR is less responsive to progestin compared with the most common PR because of (1) reduced amounts of gene transcript and (2) decreased protein activity (62). The +331G/A polymorphism is located in the promoter region of the PR gene and gives rise to

an increased synthesis of PR-B by generating an additional TATA box, thereby altering the PR-A-to-PR-B ratio (63). Given their functional consequences, it is conceivable that these genetic variants may influence the individual susceptibility for endometriosis.

Epigenetics

Epigenetic regulatory mechanisms

Classic genetics alone is not sufficient to explain the diversity of phenotypes within a population, nor does it explain how, despite the fact that their DNA sequences are identical, monozygotic twins (64) or cloned animals (65) can have different phenotypes and different susceptibilities to a disease. The concept of epigenetics may offer a logical explanation for these phenomena and could therefore be of importance in the pathogenesis of complex diseases such as endometriosis. Epigenetics refers to the study of heritable changes in gene expression capacity that occur *without* changes in the DNA sequence (66). Human cells have to manage the proper spatiotemporal expression of 23.000 genes in a broad range of cell types. Cells accomplish this feat by packaging DNA into chromatin, whose basic unit is the nucleosome, consisting of octamers of histone proteins with DNA wrapped around it. Epigenetic regulatory mechanisms modify this basic unit by the interplay of different protein complexes that interfere with chromatin configuration and subsequent accessibility of the chromatin for transcription factors, thus allowing the cell to modulate the transcriptional activity of given gene promoters. In this manner, a broad range of transcriptional activity is provided, varying from high-level expression to complete silencing. It is now widely accepted that epigenetic inheritance is an essential mechanism that allows a stable propagation of gene activity states over many cell generations. The two main epigenetic mechanisms that have emerged as critical layers of control participating in the regulation of transcription are 1) DNA methylation and 2) posttranscriptional modifications of histone proteins. In this thesis the role of DNA methylation in the pathogenesis of endometriosis has been interrogated, and will therefore be discussed in further detail below.

DNA methylation

The most studied epigenetic modification in humans is DNA methylation, which occurs at cytosines that precede a guanosine in the DNA sequence (termed CpG dinucleotides). Stretches of DNA that are rich in CpG dinucleotides are referred to as CpG islands, and are often located near the promoter region of approximately 70% genes of the human genome. In the bulk of the genome, most of the CpG dinucleotides that are not associated with CpG islands are predominantly methylated. This methylation in normal cells probably prevents chromosomal instability, translocations, and gene disruption caused by the

reactivation of transposable DNA sequences (67-69). In contrast, the cytosines within CpG islands, especially those associated with promoter regions are normally unmethylated. This lack of methylation in promoter-associated CpG islands permits expression of the gene, if the appropriate transcription factors are present, and the chromatin structure allows access to them (70). A small but significant proportion of all CpG islands become methylated during development, and when this happens the associated promoter is stably silent. Developmentally programmed CpG-island methylation of this kind is involved in genomic imprinting (the phenomenon by which only one of the two inherited copies of a gene is expressed in a parent-of-origin dependent manner), in X-chromosome inactivation (a process by which one of the two copies of the X-chromosome present in females is randomly inactivated), and in the differentiation of pluripotent embryonic stem (ES) cells. Methylation of DNA is accompanied by post-translational modifications of histone residues that modulate DNA function, regulating chromatin structure and determining the transcriptional state of the DNA wrapped around it (71). Nowadays, it is widely recognized that DNA methylation is associated with condensed heterochromatin and silencing of gene expression. This has been extensively investigated in the context of cancer research, where certain tumor suppressor genes were shown to be inactivated by promoter hypermethylation.

The mammalian DNA methylation machinery is composed of two components. The patterns of DNA methylation are established and maintained by enzymes called DNA methyltransferases (DNMTs). The crosstalk between DNA methylation and histone modifications is established by a family of proteins that contain a methyl-CpG-binding domain commonly known as MBD proteins (MBDs). These proteins are able to recognize single methylated CpG dinucleotides and recruit chromatin remodeling factors. An overview of the different DNMTs and MBDs and the mechanism by which they regulate gene expression is presented in the next paragraphs.

DNMTs

The methylation of mammalian genomic DNA is catalyzed by enzymes called DNA methyltransferases (DNMTs). In mammals, three active DNA methyltransferases—called DNMT1, DNMT3A and DNMT3B—and one related protein lacking catalytic activity, called DNMT3L, are present. A fifth protein with structural homology to the mammalian methyltransferase family is DNMT2. However, it does not exhibit particular *de novo* or maintenance methyltransferase activity in ES cells or adult somatic tissue. Its function is not yet clear, but the structure of DNMT2 suggests that it may be involved in processes such as recognition of DNA damage, DNA recombination and mutation repair. DNMTs can be divided into maintenance and *de novo* methyltransferases. DNMT1 is the major enzyme responsible for maintaining existing DNA methylation patterns during replication. It is ubiquitously and highly expressed in proliferating cells, representing the major DNA MTase activity in somatic tissues throughout mammalian development and is present only

at low levels in non-dividing cells (72). DNMT1 shows a preference for hemimethylated DNA over unmethylated DNA (73, 74), is located at the replication fork and methylates newly synthesized DNA strands directly after the replication round (75). Numerous gene targeting studies indicate a crucial role of DNMT1 in normal mammalian development, as well as in cell proliferation and survival. For example, depletion of DNMT1 has proven to be lethal, as mice deficient for DNMT1 die in mid-gestation, with significantly reduced levels of DNA methylation (76). These mice also exhibit bi-allelic expression of imprinted genes and ectopic X chromosome activation (77), indicating the importance of methylation for these processes. In addition, DNMT1 loss of function is directly linked to tumorigenesis, as demonstrated by the finding that mice with depleted levels of DNMT1 are susceptible to tumors and display chromosomal instability (78, 79).

DNMT3A and DNMT3B are *de novo* methyltransferases that are involved in the post-replicative methylation of previously unmethylated DNA. DNMT3A and DNMT3B are expressed at high levels in embryonic stem cells (ESC) but in adult somatic tissues their activity is low. The expression of DNMT3A is ubiquitous, whereas DNMT3B is expressed at very low levels in most tissues except testis, thyroid, and bone marrow (80). DNMT3B expression is profoundly increased in various tumor cell lines, indicating that this enzyme may play an important part in tumorigenesis (72, 75). Similar to DNMT1, both DNMT3A and DNMT3B are indispensable for embryonic development in mice (81). Mouse DNMT3B knockout embryos die in utero and show multiple developmental defects, whereas the DNMT3A knockout animals develop to term, but become runts and die shortly after birth.

MBDs and mechanism of gene silencing

DNA methylation is linked with transcriptional silencing of associated genes, and much effort has been invested in studying the mechanisms that underlie this relationship. So far, two basic models have evolved. In the first, DNA methylation can directly repress transcription by blocking transcriptional coactivators from binding to cognate DNA sequences (82) (Figure 1, right side). In the second, gene expression is silenced by the interplay between 5-methyl-cytosines and methyl-CpG-binding domain proteins (MBDs) (83, 84). MBDs specifically recognize and bind to methylated CpGs and recruit histone deacetylase (HDAC), either directly or via a corepressor complex. The HDAC removes acetyl groups from the lysines on the NH₂-terminal histone tails, resulting in chromatin condensation, inaccessibility of transcription factors, and transcriptional inactivation (Figure 1, left side). The process of gene silencing therefore requires the combined action of both DNMTs and MBDs.

To date, five proteins with homologous methylcytosine-binding domains have been cloned: MeCP2, MBD1, MBD2, MBD3, and MBD4. MBD proteins differ in their DNA-binding characteristics and the precise means by which they exert repressive effects, although a common theme is the recruitment of corepressors and histone deacetylase (HDAC), leading to the remodeling of chromatin. MeCP2, MBD1, and MBD2 have been

shown to recruit HDAC and function as transcriptional repressors. MBD3 is a non-DNA binding component of the Mi-2/NuRD corepressor complex (85). MBD4 is not associated with transcriptional inactivation, but has uracil DNA glycosylase activity and has been implicated in DNA repair (86).

Figure 1 Schematic representation of the two different models of gene silencing by DNMTs and MBDs. Left: Unmethylated CpGs (open spheres) are converted to 5-methylcytosines (shaded spheres) by the action of DNMTs. These are recognized by MBDs which in turn attract histone deacetylases to the site. Histone tails are deacetylated and the chromatin becomes condensed and inaccessible for transcription. Right: DNMTs directly block transcriptional coactivators from binding to cognate DNA sequences.

Endometriosis and epigenetics: the missing link?

In the last decades, numerous gene expression profiling studies have demonstrated that many genes that may promote the initiation and progression of endometriosis are consistently deregulated in tissues from endometriosis patients, such as 17-hydroxysteroid dehydrogenase type 2 (30), interleukin (IL)-1 receptor type I (87), HOXA10 (88), IL-6 (89) and aromatase (90). The fact that these aberrant expressions are seemingly quite stable in endometriosis indicates that cellular memory of some sort must be

involved. Although aberrant DNA methylation has been primarily studied in the context of cancer (70, 91), epigenetic changes have also been implicated in several complex traits such as diabetes mellitus, obesity, cardiovascular diseases and neurological disorders (92-94). Recent reports indicate a role for various epigenetic aberrations in the pathogenesis of endometriosis. The expression of certain genes that are presumably involved in the pathogenesis of endometriosis such as estrogen receptor-2 (95), steroidogenic factor-1 (96), aromatase (97) and E-cadherin (98) were shown to be regulated by promoter hyper- or hypomethylation in ectopic lesions of women with endometriosis. Furthermore, the expression of HOXA10, a gene critical for endometrial decidualization and uterine receptivity during the window of implantation, is significantly reduced in eutopic endometrium of endometriosis patients. In 2005, Wu and coworkers demonstrated that the promoter region of HOXA10 is hypermethylated in eutopic endometrium of endometriosis patients compared to eutopic endometrium of controls (99). As promoter hypermethylation is generally associated with gene silencing, this provides a plausible explanation for the observation that the expression of HOXA10 is reduced in this tissue (88, 100). The simultaneous occurrence of promoter hypermethylation and reduced HOXA10 expression has also been demonstrated in experimental animal models of endometriosis (101, 102), suggesting that HOXA10 promoter methylation may indeed constitute the mechanism through which HOXA10 is downregulated in endometriosis. The same is true for progesterone receptor B, which has been demonstrated to be undetectable in ectopic endometriotic tissues, in contrast to PR-A (42, 103). Recently, it was reported that the promoter region of PR-B, but not PR-A is hypermethylated in association with reduced expression of PR-B in epithelial cells of ectopic endometriotic lesions, suggesting that downregulation of PR-B in endometriosis is caused by promoter hypermethylation (43). Finally, overexpression of DNA methyltransferases has been implicated in the establishment of aberrant methylation patterns (104), and expression levels of DNMT1, DNMT3A and DNMT3B have been shown to be higher in ectopic endometrium compared to eutopic endometrium of both endometriosis patients and controls (105), providing support for the notion that endometriosis is, in part, an epigenetic disease.

Aims and outline of the thesis

Despite many years of hypothesis-driven research, the pathogenesis of endometriosis remains largely elusive. In addition to its description of being a hormonal disease, it is thought that environmental, genetic and epigenetic factors contribute to the initiation and progression of endometriosis. The general aim of this thesis is to gain more insight in the contribution of each of these factors to the pathogenesis of this enigmatic disease.

The first part of the thesis addresses the origin of smooth muscle like cells in deep infiltrating endometriosis and the underlying mechanism responsible for their presence. Deep infiltrating endometriosis is considered by some to be a distinct disease entity because of its distinct histological appearance resembling adenomyosis. Lesions are nodular in appearance and are largely composed of fibromuscular tissue containing smooth muscle-like cells and fibrosis, the origin of which may be the endometrium itself, or, alternatively, the local environment. In **Chapter 2**, the fibromuscular component of deep infiltrating endometriotic lesions will be characterized using immunohistochemical markers of smooth muscle differentiation. Furthermore, we will investigate the origin of the smooth muscle-like cells in endometriosis lesions in a nude mouse model. Finally, a possible causative role for TGF- β 1 in this process will be assessed by using immunohistochemical markers of active TGF- β signaling.

The second part of this thesis addresses the question whether genetic aberrations such as single nucleotide polymorphisms (SNPs), with functional consequences at the gene or protein level, in two selected candidate genes influence the susceptibility for developing endometriosis. Given the fact that TGF- β 1 has a prominent role in fibrogenesis and deep infiltrating endometriosis lesions largely consist of fibromuscular tissue, we will investigate the possible association between the presence of the -509C/T polymorphism of the TGF- β -1 gene and the risk of developing deep infiltrating endometriosis in **Chapter 3**. Because of the observed biochemical and clinical progesterone resistance in endometriosis, the prevalence of two polymorphisms of the progesterone receptor gene, PROGRINS and +331G/A, will be investigated in women with deep infiltrating endometriosis and adenomyosis of the uterine wall in **Chapter 4**. In addition to this, expression levels of PR-A and PR-B in endometriotic lesions will be correlated to the presence of either polymorphism by immunohistochemistry.

The third part of the thesis addresses the question whether epigenetic regulatory mechanisms such as DNA methylation are involved in the regulation of endometrial growth and differentiation and in differential expression of genes in endometriosis patients. In **Chapter 5**, we will assess expression levels of both components of the DNA methylation machinery, DNA methyltransferases (DNMTs) and methyl-CpG-binding domain proteins (MBDs), in [1] normal endometrium throughout the menstrual cycle and [2] eutopic and ectopic endometrium of women with endometriosis. Furthermore, hormone responsiveness of DNMT and MBD expression will be investigated in endometrial explant cultures. In **Chapter 6**, we will test the hypothesis that severe up- or

downregulation of several genes in eutopic endometrium of endometriosis patients in a previously conducted microarray study is the result of promoter methylation. Finally, in **Chapter 7**, we will discuss the implications of these findings and possible directions for future research will be given.

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Chapter 2

Fibromuscular differentiation in deeply infiltrating endometriosis is a reaction of resident fibroblasts to the presence of ectopic endometrium

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Abstract

Background

In this study, we characterized the fibromuscular (FM) tissue, typical of deeply infiltrating endometriosis, investigated which cells are responsible for the FM reaction, and evaluated whether transforming growth factor- β (TGF- β) signaling is involved in this process.

Methods

FM differentiation and TGF- β signaling were assessed in deeply infiltrating endometriosis lesions (n=20) and a nude mouse model of endometriosis 1, 2, 3 and 4 weeks post-transplantation. The FM reaction was evaluated by immunohistochemistry using different markers of FM and smooth muscle cell differentiation (vimentin, desmin, alpha-smooth muscle actin, smooth muscle myosin heavy chain). TGF- β signaling was assessed by immunostaining for its receptors and phosphorylated Smad.

Results

Deeply infiltrating endometriosis lesions contain myofibroblast-like cells that express multiple markers of FM differentiation. Expression of TGF- β receptors and phospho-Smad was more pronounced in the endometrial component of the lesions than in the FM component. In the nude mouse model, alpha-smooth muscle actin expression was observed in murine fibroblasts surrounding the lesion, but not in human endometrial stroma.

Conclusions

FM differentiation in deeply infiltrating endometriosis is the result of a reaction of the local environment to the presence of ectopic endometrium. It shares characteristics with pathological wound healing, but cannot be explained by TGF- β signaling alone.

Introduction

Endometriosis is a common benign gynecological condition characterized by the presence of endometrial glands and stroma at ectopic locations outside the uterine cavity. Deeply infiltrating endometriosis is defined as the presence of endometriosis >5 mm under the peritoneal surface and is often associated with symptoms such as dysmenorrhea, dyspareunia and pelvic pain (1). Deeply infiltrating lesions are nodular in appearance and are histologically characterized by dense tissue composed of smooth muscles and fibrosis with islands or strands of glands and stroma. In contrast to other lesion types, the major component of these nodular lesions is fibromuscular (FM) tissue rather than endometrial tissue (2). For this reason this type of lesion is often referred to as adenomyosis, and is considered by some to be a specific disease entity, distinct from peritoneal or ovarian endometriosis (3). However, it cannot be excluded that these lesions have developed from superficial peritoneal implants in the pouch of Douglas (4).

Smooth muscles are frequent components of peritoneal, ovarian, uterosacral and rectovaginal lesions but are absent in their respective unaffected sites and in eutopic endometrium of women with and without endometriosis (5). Nerve fibers trapped in these FM lesions are a significant contributor to the induction of pain symptoms in patients (6).

There is no unequivocal explanation for the presence of smooth muscle –like cells in endometriosis, and in particular deeply infiltrating endometriosis lesions. Several explanations can account for this phenomenon. First, the FM cells may result from smooth muscle metaplasia of endometrial stromal fibroblasts. It has been shown that endometrial stromal cells decidualized by progesterone *in vitro* express alpha-smooth muscle actin, a contractile microfilament that is expressed solely by smooth muscle cells, myofibroblasts and related cells (7). Smooth muscle metaplasia of endometrial stromal cells has also been described in ovarian endometriosis (8). Second, smooth muscle-like cells in deeply infiltrating endometriosis lesions originate from transdifferentiation of local tissue fibroblasts into a more contractile phenotype bearing features of smooth muscle, a phenomenon that has been extensively described within the context of tissue injury and wound healing (9). Third, the cells may have developed from remnants of the Müllerian duct system. The latter explanation is less likely, however, as smooth muscle differentiation was not restricted to deep infiltrating lesions but was observed in all lesion types (5).

All hypotheses involve differentiation of fibroblasts to myofibroblasts and, possibly, differentiated smooth muscle cells. Myofibroblasts are a unique group of smooth muscle-like fibroblasts that have acquired the capacity to neoexpress alpha-smooth muscle actin, the actin isoform typical of vascular smooth muscle cells, and to synthesize important amounts of collagen and other extracellular matrix components (10, 11). The fibroblast/myofibroblast transition is accepted as the key event in the formation of granulation tissue during wound healing and fibrotic changes. It has been shown that the

cytokine transforming growth factor- β 1 (TGF- β 1) is responsible for inducing the synthesis of alpha-smooth muscle actin in fibroblastic cells and for stimulating the production of collagen type I (12). In this respect, TGF- β 1 is the key cytokine in the evolution of lesions characterized by myofibroblast formation. This is further supported by the clinical observation that overproduction of TGF- β 1 has been implicated in the pathogenesis of several fibrocontractive diseases at various sites throughout the body, such as pulmonary fibrosis, glomerulonephritis, cirrhosis of the liver, skin scarring and peritoneal adhesion formation (13-17).

From animal studies it has become clear that transient overexpression of active TGF- β in the lung induces a chronic fibrotic response (18). Conversely, blocking TGF- β inhibits experimentally induced fibrosis in the lung, skin and liver (19-21). Given the fact that smooth muscle metaplasia and more or less extensive fibrosis can be observed in and around deeply infiltrating endometriosis lesions, we hypothesize that active TGF- β 1 signaling may be a key feature in the development of this type of endometriosis.

In this study we aim (i) to characterize the fibromuscular component of deeply infiltrating endometriotic lesions using immunohistochemical markers of smooth muscle differentiation, (ii) to investigate the origin of smooth muscle-like cells in endometriosis lesions in a nude mouse model and (iii) to assess a possible causative role for TGF- β 1 in this process by using immunohistochemical markers of active TGF- β signaling.

Materials and Methods

Patients and tissue specimens

Twenty patients with a surgical and histological diagnosis of deep infiltrating endometriosis who were operated between 1998 and 2004 in the University Hospital of Maastricht were included in the study. Deeply infiltrating endometriosis was defined as the presence of one or more deeply infiltrating lesions in the rectovaginal septum, bowel wall, vaginal wall and/or bladder wall. After evaluation of histology by a gynecopathologist, serial sections (5 μ m) were cut from paraffin embedded deeply infiltrating endometriosis lesions.

Normal endometrium for the nude mouse experiment was collected during laparoscopy in two women who had normal ovulatory cycles. Tissue was collected by transvaginal biopsy using a sampling device (Gynotec, Malden, The Netherlands) on cycle day 7 and 9 of the menstrual cycle. No gynecological pathology was found in the endometrium biopsies. The use of human endometrium was approved by the institutional ethical review committee of University Hospital Maastricht. All women gave written informed consent.

Nude mouse model

Eight female mice (Swiss v/v, Charles River, Maastricht, The Netherlands) were individually housed in autoclaved cages and bedding, in laminar flow filtered hoods. The animal room was maintained at 26 °C with a 12-h light, 12-h dark cycle, and mice were fed *ad libitum* with autoclaved laboratory rodent chow and acidified water. All handling was performed in laminar flow filtered hoods. A mixture of ketamine / xylazine (100 mg/kg ketamine and 10 mg/kg xylazine; Eurovet, Bladel, the Netherlands), subcutaneously (s.c.) injected in a volume of 0.1 ml/10g bodyweight, was used to anesthetize mice before invasive procedures, using sterile instruments. The Maastricht University ethical review committee for animal experiments approved the use of mice for this study.

At the age of 5 weeks, sterile 60-d release capsules containing 18 mg 17 β -estradiol (Innovative Research of America, Sarasota, FL) were placed s.c. in the neck of each animal. According to the manufacturer's information, capsules provide continuous release of hormone at serum concentrations of 150-250 pmol/liter in the range of physiological levels in mice during the estrous cycle (22). This stable physiological level of estrogen promotes the growth of transplanted human endometrium and eliminates intermouse differences related to various stages of the estrous cycle.

Four days after insertion of the estrogen pellet, an entrance was made to the peritoneal cavity in the midline of the lower abdomen with an 18-gauge needle, and with the help of a pipette, 10 fragments of fresh human endometrium in 200 μ l sterile PBS were inoculated intraperitoneally to mimic the situation after retrograde menstruation in women. Another entrance was made sc through the skin in the flank, and 10 fragments of fresh human endometrium were pipetted s.c. to enlarge the probability of recovery. Endometrium collected on cycle day 7 and 9 was pooled and was transplanted in all 8 mice. Two mice at a time were killed by cervical dislocation 1, 2, 3, and 4 weeks after implantation of the endometrium fragments to study the development of endometriosis lesions in time.

Analysis of endometriosis lesions in nude mice

To evaluate endometriosis lesions the abdominal skin was opened, and the abdominal s.c. region, the peritoneum and visceral organs were examined under a binocular microscope. Organs and areas suspect of endometriosis were removed, fixed in 10% buffered formalin and embedded in paraffin wax. Paraffin sections (4 μ m) were cut from the entire specimen (150-200 sections) and sections were stained with hematoxylin and eosin or used for immunohistochemistry. Histology of endometriosis lesions was evaluated by a gynecopathologist and a laboratory animal pathologist.

Characterization of FM tissue and detection of TGF- β signaling

Myofibroblasts were distinguished by antibody reaction to vimentin and desmin (intermediate filaments), alpha-smooth muscle actin (cytoskeletal element) and smooth

muscle myosin heavy chain (SM-MHC) (microfilament). A classification system of myofibroblasts is based on immunohistochemical staining of cytoskeletal filaments. Myofibroblasts that express vimentin are called V type, those that express vimentin and alpha-smooth muscle actin are called VA type, those that express vimentin and desmin are called VD type, those that express vimentin, alpha smooth muscle actin and desmin are called VAD type, and those that express vimentin, alpha-smooth muscle actin and SM-MHCs with and without desmin are called VA(D)M type. Active TGF- β signaling was detected by using antibodies directed against phosphorylated Smad2. In addition we stained for TGF- β receptors type I and II to identify the target cells in deep infiltrating endometriosis lesions and surrounding tissues.

Immunohistochemistry

A summary of the primary antibodies used and conditions applied is given in Table 1. Sections of human and mouse endometriosis lesions were fixed on Starfrost adhesive slides (Klinipath, Duiven, the Netherlands). Sections were deparaffinized in xylene and rehydrated in alcohol series prior to blocking endogenous peroxidase activity by incubation with 3% hydrogen peroxide/methanol for 20 min. After rinsing three times with phosphate-buffered saline (PBS, pH 7.2), antigen retrieval was performed (see Table 1). Sections were cooled down to room temperature and washed again three times in PBS followed by incubation with the primary antibody (room temperature, 2 h). After three PBS rinses, sections were exposed to the secondary antibody (Envision rabbit anti-mouse, ChemMate™ detection kit, DAKO, Copenhagen, Denmark) for 30 min at room temperature. Antibody binding was visualized using 3, 3' -diaminobenzidine. Sections were counterstained mildly with hematoxylin, dehydrated and mounted in Entellan for light microscopy. Negative control slides for the monoclonal antibodies were incubated with mouse immunoglobulin (Ig)Gs of the same class and same dilution as the primary antibodies. Negative control slides for the rabbit polyclonal antibodies (pAbs) were incubated with rabbit IgG at the same dilution as the pAbs.

Evaluation of immunostaining in human endometriotic tissues

The percentage of stained cells (0, 0-10, 10-50, >50%) and the intensity of staining (absent, weak, moderate, strong) were determined (0, 1, 2 or 3 for each variable) for the entire lesion with respect to (i) visceral smooth muscle (if present), (ii) connective tissue not comprising part of fibromuscular reaction, (iii) fibromuscular tissue surrounding endometriosis lesions, (iv) endometrial stromal cells and (v) endometrial epithelial cells.

Table 1 Primary antibodies and conditions used for immunohistochemistry of human and mouse endometriosis lesions

Antibody	Species	Catalog number	IgG class	Dilution	Manufacturer	Antigen retrieval
Vimentin	Mouse	M0725	IgG1k	1:100	DAKO, Copenhagen, Demark	Citrate (pH 6.0)
α - smooth muscle actin	Mouse	M0851	IgG2ak	1:500	DAKO, Copenhagen, Denmark	Tris EDTA (pH 9.0)
Desmin	Mouse	MUB0400	IgG2b	1:700	MUbio products BV, Maastricht, the Netherlands	Citrate (pH 6.0)
SM-Myosin heavy chain	Mouse	260850-1	IgG1k	1:3000	Northstar Bioproducts, East Falmouth, MA, USA	Tris EDTA (pH 9.0)
TGF- β receptor type I	Rabbit	sc-398	Not specified	1:2000	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Citrate (pH 6.0)
TGF- β receptor type II	Rabbit	sc-220	Not specified	1:3000	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Citrate (pH 6.0)
Phosphorylated Smad2	Rabbit	3101	Not specified	1:1000	Cell Signaling, Danvers, MA, USA	Citrate (pH 6.0)

A staining index (SI, ranging from 0 to 9) was calculated by multiplying categorized parameters. Two different observers (P.G.G. and K.J.A.F.K) performed evaluation of immunostaining in a blinded fashion. Both observers scored the sections once. The mean of these two observations was used for analyses. The level of inter-observer agreement was determined by calculating kappa statistics for ordinal variables and showed that concordance between the two observers was adequate with respect to the percentage of stained cells (κ 0.73), intensity of the staining (κ 0.86) and SI (κ 0.76).

Results

Human deeply infiltrating endometriosis lesions

All twenty paraffin embedded deeply infiltrating endometriosis tissue specimens contained connective tissue, endometriosis lesions consisting of endometrial epithelium and endometrial stroma and fibromuscular tissue surrounding endometriosis lesions (Figure 1). Seventeen out of 20 paraffin-embedded tissue samples contained visceral smooth muscle. Immunohistochemistry results from patient tissue did not differ between lesions obtained from rectovaginal septum, bowel wall, vaginal wall or bladder wall. To investigate the immunophenotype of the lesions and surrounding tissues, sections were stained for vimentin, alpha-smooth muscle actin, desmin and smooth muscle myosin (Figures 1 and 2). Representative photographs of the negative controls for the various immunohistochemical staining procedures are presented in Figure 3.

Figure 1 Representative photographs of deep-invasive endometriotic lesions stained with antibodies against vimentin, alpha-smooth muscle actin (ASMA), desmin and smooth muscle myosin heavy chain (SM- myosin). Vimentin staining is strong in the stroma (Str) of the ectopic endometrium, whereas the cells surrounding the lesion (FM) are positive for ASMA. Some areas (M) are also positive for desmin and SM-myosin; most likely resident smooth muscle cells. Scale bar = 100 μ m. (For color figure see page 145)

Figure 2 Staining indices for vimentin, ASMA, desmin and SM-myosin in the different tissue components of endometriotic lesions and host environment.
■ visceral smooth muscle, ■ connective tissue, □ fibromuscular tissue, ■ endometrial stroma, ■ endometrial epithelium.

Figure 3 Representative photographs of the negative controls for the vimentin, ASMA, desmin, SM-myosin, TGF- β receptor 1 and 2 and phosphorylated Smad immunostainings. Scale bar = 100 μ m. (For color figure see page 146)

Connective tissue fibroblasts

As a reference to assess the extent of fibromuscular differentiation close to the lesions, we evaluated the immunophenotype of connective tissue fibroblasts of the submucosal connective tissue of the large bowel in patients with deep infiltrating endometriosis lesions. An example is presented in Figure 4. The intermediate filament vimentin was strongly expressed in connective tissue fibroblasts (mean SI 8.7 ± 0.8 , Figure 2). In some tissue samples, connective tissue fibroblasts showed weak desmin expression (mean SI 2.0 ± 2.6), whereas expression of myosin heavy chain and alpha-smooth muscle actin was completely absent in connective tissue fibroblasts of all specimens.

Figure 4 Immunostaining of vimentin, ASMA, desmin and SM-myosin in submucosal connective tissue of the large bowel. Scale bar = 100 μ m. (For color figure see page 147)

Endometrial stroma

Endometrial stromal cells in deeply infiltrating endometriosis lesions strongly express vimentin (mean SI 7.2 ± 2.3). Weak expression of alpha-smooth muscle actin (mean SI 1.3 ± 0.8) and desmin (mean SI 2.0 ± 1.2) could be observed, mostly localized in endothelial cells of blood vessels within the lesions (Figures 2 and 5). SM-MHC expression (mean SI 0.3 ± 0.6) was very weak and could only be observed in a small minority of lesions (Figures 2 and 5).

Fibromuscular tissue

Cells comprising part of the fibromuscular reaction around endometriosis lesions also strongly express vimentin (mean SI 7.3 ± 1.9). As opposed to fibroblasts in connective tissue, these cells abundantly express alpha-smooth muscle actin (Figures 2 and 6; mean SI 7.0 ± 1.7), thereby demonstrating their myofibroblastic nature. Within the regions that stain positive for alpha-smooth muscle actin, focal areas showing moderate to strong expression of smooth muscle differentiation markers desmin and myosin heavy chain (mean SI 3.3 ± 1.6 and 3.4 ± 1.6 , respectively) can be observed (Figure 6).

Figure 5 Higher magnification photographs of an endometriotic lesion in the sigmoid, with fibromuscular tissue, stained for vimentin, ASMA, desmin and SM-myosin. Str = stroma in ectopic lesion, FM = fibromuscular tissue. Scale bar = 100 μ m. (For color figure see page 147)

Figure 6 Difference in fibromuscular tissue and structures that are most likely resident smooth muscle cells. The sections were stained for ASMA, desmin and SM-myosin. Str = stroma in ectopic lesion, FM = fibromuscular tissue, M = visceral muscle. Scale bar = 100 μ m. (For color figure see page 148)

Visceral smooth muscle

Visceral smooth muscle shows weak vimentin expression (mean SI 1.3 ± 1.6) and abundant generalized expression of desmin (mean SI 7.9 ± 0.7), myosin heavy chain (mean SI 7.1 ± 1.0) and alpha-smooth muscle actin (mean SI 6.3 ± 0.6), all markers of smooth muscle differentiation (Figures 2 and 7). Sometimes endometriotic lesions are present in the visceral muscle layer (Figure 7).

TGF- β signaling

Smad2 phosphorylation was closely associated with the presence of TGF- β receptors type I and II. Expression of TGF- β receptor I, TGF- β receptor II and phosphorylated Smad2 was most pronounced in endometrial epithelium (mean SI 6.5 ± 1.2 / 6.8 ± 1.6 / 7.9 ± 1.3 , respectively) followed by endometrial stroma (mean SI 4.0 ± 1.0 / 3.7 ± 1.0 / 6.8 ± 1.4 , respectively), visceral smooth muscle (mean SI 4.5 ± 1.2 / 3.4 ± 0.9 / 6.0 ± 2.1 , respectively) and connective tissue (mean SI 3.8 ± 1.1 / 2.3 ± 1.6 / 5.0 ± 2.0 , respectively) and was least pronounced in cells comprising part of the fibromuscular reaction (mean SI 1.9 ± 0.7 / 1.5 ± 0.8 / 4.6 ± 2.2 respectively) (Figures 8 and 9).

Figure 7 A lesion in the visceral muscle of the colon wall. The stroma is positive for vimentin and ASMA, whereas the muscle cells are positive for ASMA, desmin and SM-myosin. Scale bar = 100 μ m. (For color figure see page 148)

Figure 8 Staining indices for TGF- β receptor 1 and 2 and phosphorylated Smad in the different tissue components of endometriotic lesions and host environment.

■ visceral smooth muscle, ■ connective tissue, □ fibromuscular tissue, ■ endometrial stroma, □ endometrial epithelium.

Figure 9 Representative photographs of immunostaining for TGF- β receptor 1 and 2 and phosphorylated Smad in deeply infiltrating endometriosis. Str = stroma in ectopic lesion, FM = fibromuscular tissue, Gl = gland in ectopic lesion. Scale bar = 100 μ m. (For color figure see page 149)

Mouse endometriosis lesions

Endometriosis lesions were identified in all mice, except for one mouse that was sacrificed after 2 weeks. Most lesions were found at the s.c. injection sites, some lesions were present on the peritoneum near the umbilical region. All lesions consisted of endometrial glands and stroma. For immunohistochemical analysis the subcutaneous lesions were used. Cells of human origin could be distinguished from mouse cells by positive staining for the human-specific vimentin antibody (Figure 10).

Figure 10 Vimentin and ASMA expression in endometriotic lesions generated through the subcutaneous transplantation of human endometrium in nude mice. Lesions were collected weekly for four weeks. The vimentin antibody is human-specific, which demonstrates that the ASMA expression is strongly enhanced in the host cells. Negative controls for the vimentin and ASMA stainings are shown in the inserts. Scale bar = 100 μ m. (For color figure see page 150)

One week after tissue inoculation, alpha-smooth muscle actin was highly expressed in the mouse cells directly surrounding the lesion (Figure 10) but not in the human endometrial cells or in connective tissue fibroblasts remote from the lesion. Two weeks after inoculation, alpha-smooth muscle actin expression in mouse cells slightly decreased. After three and four weeks alpha-smooth muscle actin expression progressively declined but remained visible as a thin sheath directly adjacent to and surrounding the endometrial glandular epithelium. The number of endometrial stromal cells appeared to decrease at the same time collagen deposition became apparent in a circular pattern surrounding the lesion after three weeks.

Discussion

Deeply infiltrating endometriosis is characterized by the existence of nodular lesions largely composed of FM tissue. In this study we show that FM tissue surrounding endometriosis lesions contains myofibroblastic cells that, in addition to alpha-smooth muscle actin, express multiple markers of smooth muscle differentiation such as desmin and SM-MHC.

Our findings support the contention that the formation of deeply infiltrating endometriosis lesions shares characteristics with pathological wound healing. During the initial phase of normal wound healing, alpha-smooth muscle actin is highly expressed by myofibroblasts in granulation tissue, thereby effectuating the necessary wound contraction. The contractile activity of myofibroblasts is terminated when the tissue is repaired: alpha-smooth muscle actin expression decreases and myofibroblasts disappear through massive apoptosis (23). In pathological fibrotic phenomena however, this wave of apoptosis is lacking and alpha-smooth muscle actin expression persists in the tissue myofibroblasts in addition to the expression of other markers of smooth muscle differentiation such as desmin and SM-MHC (24).

The presence of smooth muscles in endometriosis lesions is not a new finding: Anaf *et al.* demonstrated alpha-smooth muscle actin positivity in areas surrounding peritoneal, ovarian, uterosacral and rectovaginal endometriosis lesions (5). Smooth muscles were absent in the unaffected peritoneum and in the eutopic endometrium of women with and without pelvic endometriosis. In line with the induction theory (25), these authors speculated that the smooth muscle component of endometriosis might result from the capacity of the secondary Müllerian system to differentiate into both smooth muscle cells and endometrial glands and stroma. Alternatively, endometrial cells arriving through retrograde transplantation at ectopic sites could undergo smooth muscle metaplasia, or induce the surrounding tissue to undergo smooth muscle metaplasia. With the aid of a nude mouse model we were able to demonstrate that as soon as one week after inoculation with human endometrium, alpha-smooth muscle actin expression is induced in the surrounding murine fibroblasts, whereas no expression was observed in the human

cells. These findings strongly suggest that the presence of smooth muscle-like tissue in deeply infiltrating endometriosis lesions is accounted for by a reaction of the local environment to the presence of ectopic endometrium rather than smooth muscle metaplasia of the ectopic endometrium itself.

This phenomenon mimics what is frequently observed in malignancies. Many epithelial tumors are also characterized by the presence of an “activated” stroma consisting of fibroblastic and myofibroblastic cells that produce collagen and extracellular matrix components, a phenomenon that is referred to as the stroma reaction or “desmoplastic reaction” (26). Desmoplasia is considered a response of the resident stromal fibroblasts of the host environment to inductive stimuli exerted by tumor cells, such as diffusible factors, extracellular matrix and/or direct cell-to-cell contacts. The reciprocal interactions between the tumor cells and resident fibroblasts potentiate tumor growth, stimulate angiogenesis and induce fibroblasts to undergo differentiation into myofibroblasts. Although endometriosis cannot be regarded as a *bona fide* neoplasm, it displays certain important characteristics of malignant tumor growth such as invasion of the extracellular matrix (27) and the acquisition of its own blood supply (28). Therefore, it is conceivable that a similar process may take place in the evolution of smooth muscle-containing endometriotic lesions. However, a limitation of the present study is constituted by the fact that the endometrial tissue used for the animal study was sampled from only two women and the experiment was carried out only once in a relatively small number of animals. The possibility that these results occurred by chance can therefore not be ruled out completely.

We hypothesized that prolonged TGF- β signaling is involved in the development of fibromuscular tissue in deep infiltrating endometriotic lesions. It has been shown that the cytokine TGF- β 1 is responsible for inducing the synthesis of alpha-smooth muscle actin in fibroblastic cells and for stimulating the production of collagen type I (12), and overproduction of TGF- β 1 has been implicated in the pathogenesis of disorders characterized by fibrosis (13-17). In this respect, TGF- β 1 is a key cytokine in the evolution of lesions characterized by myofibroblast formation. TGF- β 1 is expressed in the human endometrium throughout the menstrual cycle, it is regulated by ovarian steroids (29, 30), and could be responsible for the induction of the FM reaction in the fibroblasts of the host environment. Surprisingly however, the expression of the marker of active TGF- β signaling, phosphorylated Smad 2, was consistently lower in myofibroblasts of the FM tissue compared with surrounding tissues. Consistent with these findings, we found that the expression of the receptors for TGF- β was most pronounced in the endometrial tissue of the lesion, and not in the surrounding fibromuscular tissue. Most TGF- β signaling apparently occurs in the endometrial tissue and not in the local host cells.

An important factor which may contribute to the differentiation and maintenance of the myofibroblast phenotype could be mechanical strain (31). Deeply infiltrating endometriosis lesions are often found in areas subjected to continuous or intermittent mechanical tension: the bowel wall and rectovaginal septum repeatedly stretch and relax

as a result of peristalsis and the passage of feces. Frequent mechanical stress could constitute an important determinant in the development of deep infiltrating endometriosis lesions. In rat models for wound healing in the skin, Hinz *et al.* showed that mechanically inflicted tension induced alpha-smooth muscle actin expression in fibroblasts in the wound; relief of the mechanical pressure resulted in disappearance of the actin filaments which preceded a decrease in TGF- β 1 levels (32). These results indicate that TGF- β 1 alone may not be sufficient to maintain myofibroblast differentiation, and that a mechanical stimulus is equally important. Shi *et al.* showed in injured porcine arteries that after fourteen days of coexpression, TGF- β 1 expression disappeared, whereas alpha-smooth muscle actin expression in the neointima of injured porcine arteries remained high up to 90 days (33). As arteries are also subject to mechanical strain due to the high intravascular pressure, it is plausible that this is responsible for the continued alpha-smooth muscle actin expression.

Alternatively, the excessive fibromuscular reaction could be explained by the fact that ectopic endometrium arriving through repeated retrograde menstruation induces a chronic inflammatory response in the peritoneal cavity. It has been shown that the peritoneal fluid of women with endometriosis is marked by increased inflammation, including increased concentration of white blood cells and macrophages, and increased activation status of these macrophages (34, 35). In association with the activated state of these macrophages, an increase in the release of macrophage-derived cytokines such as TGF- β 1 has been demonstrated in the peritoneal fluid of women with endometriosis (36-38). Overproduction of TGF- β 1 by activated macrophages in peritoneal fluid may therefore contribute to a microenvironment that favors the formation of fibromuscular tissue surrounding endometriosis lesions.

In conclusion, in this study we show that the FM cells surrounding deep infiltrating endometriosis lesions express multiple markers of FM differentiation, resembling the situation in pathologic wound healing and fibrocontractive diseases. In a nude mouse model we showed that alpha-smooth muscle actin expression is induced in the host tissue after implantation of human endometrial fragments, suggesting that the presence of smooth muscle-like tissue in endometriosis lesions is the result of a reaction of the local environment to the presence of ectopic endometrium rather than smooth muscle metaplasia of the ectopic endometrium itself. Based on our observations it is not likely that TGF- β 1 signaling alone is sufficient to account for the excessive FM tissue in deep infiltrating endometriosis lesions. In this respect, the presence of mechanical tension and increased inflammatory activity in peritoneal fluid of women with endometriosis may be important contributing factors in the establishment of myofibroblast-containing deep infiltrating endometriosis lesions.

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Chapter 3

Transforming growth factor β 1 gene polymorphism -509C/T in deep infiltrating endometriosis

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Abstract

Deep infiltrating endometriosis is characterized by the presence of nodular lesions largely composed of fibromuscular tissue. Transforming growth factor beta 1 (TGF- β 1) is the cytokine most causatively associated with disorders characterized by fibrosis throughout the body. Therefore, the hypothesis was tested that mechanisms increasing the fraction of biologically active TGF- β 1, such as TGF- β 1 gene polymorphisms, lead to an increased risk of developing deep infiltrating endometriosis. The frequency of the -509C/T polymorphism of the TGF- β 1 gene was tested in women with deep infiltrating endometriosis (n=72), gynecological patients without symptoms of endometriosis (n=95) and healthy females (n=93). Detection of the -509C/T polymorphisms was performed using PCR-restriction fragment length polymorphism analysis. We did not observe statistically significant differences in the frequency of the -509C/T polymorphism between the groups. Our study does not support an association between the -509C/T polymorphism of the TGF- β 1 gene and an increased risk of deep infiltrating endometriosis.

Introduction

Endometriosis is a commonly occurring, complex disease characterized by the presence of endometrial glands and stroma at ectopic locations outside the uterine cavity. The disease can manifest itself as superficial peritoneal lesions, ovarian endometriotic cysts and as deep infiltrating lesions, most often in the rectovaginal space. Many women with patent tubes harbor spots of peritoneal endometriosis without having any symptoms. In these women, superficial peritoneal lesions are often referred to as the phenomenon endometriosis. On the other hand, deep infiltrating lesions are considered to be the disease endometriosis, since they often lead to symptoms such as severe dysmenorrhea, dyspareunia, and pelvic pain. However, the pathogenesis of deep infiltrating endometriosis is still poorly understood and surrounded by controversy. Theories that have been developed on this subject either favor the argument that deep endometriosis develops from superficial peritoneal implants that infiltrate into the pouch of Douglas and the rectovaginal septum (1), whereas others suggest that deep infiltrating lesions arise as a result of metaplasia of Müllerian remnants, implying that deep infiltrating endometriosis is a specific disease entity, distinct from other types of endometriosis (2, 3).

Deep infiltrating endometriosis is defined as the presence of endometriosis more than 5 mm under the peritoneal surface (4), and often takes the form of a nodular lesion. A key feature of these deep infiltrating lesions is that, in contrast to superficial peritoneal implants, their major component is fibromuscular tissue rather than endometrial tissue. More or less extensive fibrosis has been demonstrated in nearly 100% of rectovaginal deep infiltrating endometriotic lesions (5). Endometriosis is generally considered to be a complex, polygenic disease with a multifactorial etiology. Based on biological plausibility, numerous candidate genes and their allelic variations were analyzed for their possible involvement in the pathogenesis of endometriosis (6-10). So far, this has yielded inconsistent results. A gene that may be of particular importance with respect to the pathogenesis of deep infiltrating endometriosis, is the transforming growth factor β 1 (TGF- β 1) gene. TGF- β 1 is the cytokine most causatively associated with disorders characterized by fibrosis (11). In the embryo, its fibrogenic effects are critical during both organogenesis and morphogenesis, whereas in the adult, they are central in wound healing and tissue repair. TGF- β 1 exerts its fibrogenic effects by inducing matrix deposition in mesenchymal cells in culture by promoting expression of extracellular matrix (ECM) genes, suppressing the activity of enzymes that degrade ECM (such as matrix metalloproteinases), enhancing fibroblast proliferation and inducing the myofibroblast phenotype.

Although TGF- β 1 is essential for wound healing, overproduction of TGF- β 1 has been implicated in the pathogenesis of several fibrotic diseases at various sites throughout the body, such as pulmonary fibrosis, glomerulonephritis, cirrhosis of the liver, skin scarring and peritoneal adhesion formation (12-15). Treatment of fetal wounds with TGF- β 1 promotes wound closure and scarring, supporting the notion that TGF- β 1 promotes

fibrosis *in vivo* (16). Conversely, the cutaneous or intraperitoneal application of neutralizing antibodies against TGF- β 1 in rats decreased scar formation at the site of dermal injury, as well as intra-abdominal adhesions (17, 18).

High levels of TGF- β 1 have also been linked to the pathogenesis of endometriosis. It has been demonstrated that peritoneal fluid levels of TGF- β 1 are elevated in women with endometriosis and women with pelvic adhesions compared to women with a normal pelvis (19-21). However, despite the positive association between TGF- β 1 concentrations in peritoneal fluid and endometriosis, a direct causal relationship remains to be demonstrated.

Given the pivotal role of TGF- β 1 in various fibrotic disorders and the amount of fibromuscular tissue present in deep infiltrating endometriotic lesions, we hypothesized that mechanisms increasing the fraction of biologically active TGF- β 1, such as TGF- β 1 gene polymorphisms, lead to an increased risk of developing deep infiltrating endometriosis. Past research has shown that the -509C/T polymorphism of the TGF- β 1 gene is associated with a significant increase of mean TGF- β 1 plasma concentration, to the extent that almost 10% of the genetic variance in total TGF- β 1 plasma concentration could be attributed to the -509C/T polymorphism (22). One study has demonstrated a strong association between the presence of the -509T polymorphic allele and endometriosis (23). However, in their study there was no specific assessment of patients with deep infiltrating lesions although this study included patients with moderate/severe endometriosis according to the revised American Fertility Society classification.

The aim of the current study was to investigate the possible association between the presence of the -509C/T polymorphism and the risk of developing deep infiltrating endometriosis. Two groups of women without symptomatic endometriosis served as controls.

Materials and Methods

Patients and tissue specimens

Patients with a surgical and histological diagnosis of deep infiltrating endometriosis who were operated between 1998 and 2004 in the University Hospital of Maastricht (n=72) were included in the study. Deep infiltrating endometriosis was defined as the histologically proven presence of one or more deep infiltrating lesions in the bowel wall, vaginal wall, bladder wall and/or rectovaginal septum. The first control group consisted of gynecological patients without symptoms of endometriosis who underwent a hysterectomy for benign indications such as prolapse, pelvic pain or menstrual cycle disorders (gynecological controls, n=95). Patients with uterine fibroids and postmenopausal women were excluded from this group. A second group of controls was taken from a DNA-control panel derived from female subjects who had been referred to

the Clinical Genetics department for molecular testing of various disease genes. Only subjects that were tested negative for these diseases were included in the control panel, as they can be regarded as healthy, random controls (population controls, n=93). Both patients and controls originated from the same geographic region of the Netherlands.

DNA isolation

In patients with deep infiltrating endometriosis and the gynaecological control group the analyses were performed on DNA isolated from paraffin-embedded archival tissue. After reviewing all paraffin-embedded tissue specimens for the presence of endometriotic lesions, five consecutive sections of 20 μ m were cut from each tissue sample. Genomic DNA isolation was performed using proteinase K (Qiagen, Hilde, Germany) digestion in combination with the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA), following the recommendations of the manufacturer.

The analyses of the population control group were performed on DNA isolated from blood. Genomic DNA was isolated from 8 ml whole blood (EDTA tubes) using the Wizard Genomic DNA Purification Kit (Promega, Leiden, the Netherlands) following the manufacturer's protocol.

-509C/T detection

Detection of the -509C/T polymorphism (NCBI SNPs database number: rs1800469) was performed using restriction fragment length polymorphism (RFLP) analysis. From the patients with deep infiltrating endometriosis and the gynaecological controls only paraffin-embedded archival tissue was at our disposal, which is known to yield lower amounts of DNA of inferior quality compared to DNA isolated from blood or fresh tissue. Therefore, a nested PCR approach was used to obtain a higher sensitivity. We were able to successfully genotype 68/72 patients with deep infiltrating endometriosis and 91/95 gynecological controls. In the population control group DNA isolated from blood was used for analyses, and we were able to successfully genotype all 93 individuals in this group.

All PCR's were performed in a final volume of 20 μ l, containing 100 ng of genomic DNA, 50 nM of each primer, 0.1 mM of dNTP, 1 unit of Taq DNA polymerase and 1 x buffer with 1.5 mM MgCl₂. A summary of specific primers and cycling conditions is listed in Table 1. Upon digestion with *DdeI* (Promega) the 117 bp fragment generated with the nested PCR contained one restriction site (generating two fragments of 72 and 45 bp) if derived from the most common allele -509C, and no restriction site (generating one fragment of 117 bp) if derived from the polymorphic allele -509T. Electrophoresis was performed with the use of a 3% agarose gel stained with Gelstar (Cambrex, ME, USA) for visualisation of the PCR products (Figure 1).

Table 1 Primer sequences and conditions for determination of transforming growth factor $\beta 1$ gene polymorphism –509C/T

-509 C/T	Forward	Reverse	Annealing temperature (°C)	No of cycles	Size of amplicon (bp)
Flank primers	5'GAGTGACAGGAGGCTGCTTAGC	5'GTGTGGGTCACCAGAGAAAGAG	55	25	188
Nested primers	5'GGAGAGCAATTCTTACAGGTGTCT	5'CGAAAGGAGGGTCTGTCAACA	55	35	117

In all three groups only women of reproductive age were included (range: 20-50 years old). All samples were analyzed in duplo for both polymorphisms. In case of a discrepancy between the first and second result, the analysis was repeated. If the result remained inconclusive, the sample was omitted from the analysis.

Statistical analysis

Hardy-Weinberg equilibrium for the –509C/T polymorphism was assessed in each group by comparing the Pearson's goodness of fit with a chi square distribution with one degree of freedom (Institute of Human Genetics, Munich, Germany: <http://www.ihg.gsf.de/>). Allele and genotype frequencies were compared between groups using Fisher's exact test. The odds ratio (OR) was used to measure the strength of the association between the frequencies of allele and genotype and deep infiltrating endometriosis. Calculations were done using SPSS software (version 11.5). All P values were two-tailed and 95% CI's were calculated. A P value <0.05 was considered to be statistically significant.

Results

The summarized TGF- $\beta 1$ genotyping data are presented in Table 2. The genotype distribution for the –509C/T polymorphism did not differ from Hardy-Weinberg equilibrium in both patient and referent groups. Proportions of –509C/C homozygote, –509C/T heterozygote and –509T/T homozygote genotypes were 50.0%, 36.8% and 13.2% in the group of women with deep infiltrating endometriosis, 53.8%, 34.1% and 12.1% in gynecological controls and 44.1%, 47.3% and 8.6% in population controls. Frequencies for the most common allele -509C and the polymorphic allele –509T were 68.4% and 31.6% in women with deep infiltrating endometriosis, 70.9% and 29.1% in gynecological controls and 67.7% and 32.3% in population controls. No statistically significant differences in allele or genotype frequencies were found between patients with deep infiltrating endometriosis and either control group.

Figure 1 Restriction fragment length polymorphism analysis of the transforming growth factor beta1 gene polymorphism -509C/T. wt: patient not carrying the -509T polymorphic allele (-509C/C individual); he: heterozygous -509T carrier (-509C/T individual); ho: homozygous -509T carrier (-509T/T individual)

Discussion

Deep infiltrating endometriosis is characterized by the existence of nodular lesions that are largely composed of fibromuscular tissue. In analogy to pathologic processes such as liver, kidney, and cardiac fibrosis, the establishment of deep infiltrating endometriotic lesions may also be regarded as a fibrotic phenomenon. Since the cytokine TGF- β 1 has long been recognized to be a central mediator in tissue fibrosis, it was hypothesized that the development of this type of lesions is associated with an increased fraction of biologically active TGF- β 1.

However, despite the fact that the -509C/T polymorphism of the TGF- β 1 gene is associated with a significant increase in TGF- β 1 serum concentration, in the present study we show that this polymorphism was not associated with an increased risk of deep infiltrating endometriosis. Our results contradict the ones of Hsieh et al. (23), who found an association between the presence of the -509T polymorphic allele and moderate/severe forms of endometriosis. It is known that in case of genetic association studies, one of the major problems causing false-negative results derives from an inadequate sample size, which leads to insufficient statistical power.

Table 2 Genotype and allele frequencies of the transforming growth factor β 1 gene polymorphism -509C/T in women with deep infiltrating endometriosis and controls

-509C/T	Deep infiltrating endometriosis (n=68, n (%))	Gynecological controls (n=91, n (%))	Population controls (n=93, n (%))
Genotypes			
-509C/C	34 (50.0)	49 (53.8)	41 (44.1)
-509C/T	25 (36.8)	31 (34.1)	44 (47.3)
-509T/T	9 (13.2)	11 (12.1)	8 (8.6)
-509C/T + TT	34 (50.0)	42 (46.2)	52 (55.9)
Alleles			
-509C	93 (68.4)	129 (70.9)	126 (67.7)
-509T	43 (31.6)	53 (29.1)	60 (32.3)

n = number of patients that could be genotyped successfully

However, a power calculation reveals that the sample size used in the present study is sufficient to detect a potential increase in risk (Odds Ratio, OR) for polymorphic allele-carriers to develop the disease (in this case deep infiltrating endometriosis) of at least 2.8 with a statistical power of 80% and an α of 0.05. Furthermore, when performing a power calculation based on the increase in risk found by Hsieh et al. (OR 3.6), statistical power rose to 95% at the 5% confidence level with the present sample size. It is therefore unlikely that the discrepancy between our results and the results obtained by the group of Hsieh is based on the sample size of the present study being too small. On the other hand, the study by Hsieh presents several major limitations, which lead us to suggest that their conclusions should be handled with caution. These authors found proportions of C/C homozygote, C/T heterozygote and T/T homozygote genotypes of 49.3%, 61.3% and 29.4% in endometriosis patients and 1.3%, 58.5% and 0% in controls. These proportions deviate significantly from the Hardy-Weinberg equilibrium (HWE) in the control group. HWE includes a series of assumptions regarding the population tested, such as the large-size (i.e. no genetic drift should occur), the absence of new mutations, migration or natural selection, and the occurrence of random mating. Deviations from the HWE are due either to violation of these assumptions, indicating that the tested group is not representative of the general population, or may point to genotyping errors. Violation of the HWE in controls therefore lead to false genetic associations with a particular disease or pathological condition (24).

Another pitfall of this study is the selection of the control group. As stated by Zondervan and coworkers (25), appropriate selection of cases, and in particular of controls, is extremely important because this may have a profound effect on the results of candidate gene studies. In the study by Hsieh and coworkers, the authors reportedly confirmed the non-endometriosis status of their control group during either caesarean section or diagnostic laparoscopy. However, in this way one is very likely to miss deep infiltrating endometriotic lesions, as they are often not visible above the peritoneal surface. This is especially likely during caesarean section, when overview of the pelvis is impaired because of the enlarged uterus and sometimes large quantities of blood. Moreover, the progestagen-rich milieu provided by gestation may cause lesions that were present before pregnancy to substantially decrease in size, possibly to the extent that they are no longer visible at the time of (operative) delivery. It is therefore conceivable that the control group in this study was biased with respect to the occurrence of non-recognized endometriosis.

In order to minimize this type of bias, we included two control groups in the present study. The first one consisted of a well-defined group of gynecological patients without symptoms of endometriosis who underwent a hysterectomy for benign indications such as pelvic pain or heavy or irregular menstrual bleeding. Moreover, as recommended by Zondervan and coworkers (25), a second, general population-based control group consisting of healthy women of reproductive age was included in the study, as this group of women is the most representative of the source population from which cases were derived. In our opinion, the fact that we found no differences in the prevalence of the –

509C/T polymorphism between patients with deep infiltrating endometriosis and either of the two control groups makes our results even more robust.

Finally, the study by Hsieh and coworkers (23) focused on patients with moderate/severe endometriosis according to the revised American Fertility Society criteria. However, this classification system is not particularly suitable for the purpose of defining the severity of endometriotic disease, since these criteria were principally developed to categorize women according to the probability of conceiving and do not correlate well with the presence of symptoms such as pelvic pain. Recently, Holt and Weiss have proposed a standard definition of definite (i.e. severe) and possible (i.e. less severe) endometriotic disease in order to render study results comparable between each other (26). In the present study, symptomatic women with histologically proven deep infiltrating endometriosis were included, which is in accordance with the criteria for definite disease as proposed by Holt and Weiss.

Although we did not find a significant association between the presence of the -509C/T polymorphism and deep infiltrating endometriosis, a possible role of TGF- β 1 and its overproduction in the pathogenesis of this disease entity cannot be excluded. A possible explanation could be that there is excess production of TGF- β 1 in these patients, but this takes place in the local peritoneal environment rather than in serum. TGF- β 1 is a major secretory product of macrophages and is detectable in peritoneal fluid. It has been shown that peritoneal fluid of women with endometriosis contains an increased number and activation of macrophages (27, 28). Endometriosis produces a localized inflammatory response. As a result, macrophages are attracted through chemotaxis and extravasate through small pores in the vessel wall into the peritoneal cavity where they perform their phagocytic and secretory functions. The higher number and activation of macrophages in the peritoneal cavity of women with endometriosis is likely to be accompanied by an increase in macrophage-derived cytokines such as TGF- β 1.

This contention is supported by the fact that three studies conducted on this subject consistently reported a significant increase of peritoneal fluid levels of TGF- β 1 in women with endometriosis compared to controls (19-21). However, it must be emphasized that the increased presence of macrophages and cytokines such as TGF- β 1 does not prove their involvement in the development and maintenance of endometriotic implants. These factors were investigated in women who had already developed endometriosis and may be therefore have been a consequence rather than a cause of the disease.

With respect to TGF- β 1 levels in serum of women with endometriosis, results are less equivocal. One study reported a significant increase of TGF- β 1 levels in women with endometriosis (n=26) compared with controls (n=5) (20). However, although the difference is statistically significant, these results should be handled with caution because of the disproportionally low number of controls compared to cases. Another study compared serum and peritoneal fluid levels of TGF- β 1 between women with deep infiltrating endometriosis and women with superficial endometriosis (29). The results did not show significant differences between the groups with respect to TGF- β 1 levels in

blood or in peritoneal fluid. However, with a sample size too small to detect small but significant differences, this study was significantly underpowered. The question whether serum levels of TGF- β 1 are indeed elevated in women with endometriosis therefore cannot be answered conclusively.

In conclusion, we have shown that the presence of the TGF- β 1 gene polymorphism -509C/T is not associated with an increased risk of developing deep infiltrating endometriosis or adenomyosis in the uterine wall. Future research should be aimed at providing a more definite answer to the question if elevated levels of TGF- β 1 in serum or peritoneal fluid are in fact causally related to the presence of endometriosis, or if they represent a mere consequence of the disease.

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Chapter 4

Progesterone receptor polymorphism +331G/A is associated with a decreased risk of deep infiltrating endometriosis

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Abstract

Background

Alterations in the progesterone receptor (PR) are considered a risk factor for the development of endometriosis. In this study the frequencies of the PROGENS and +331G/A polymorphisms of the PR gene were determined in deep infiltrating endometriosis, and correlated with the expression of the PR protein.

Methods and results

The frequencies of the PR polymorphisms were determined in women with deep infiltrating endometriosis (n=72), women with adenomyosis in the uterine wall (n=40), gynecological patients without symptomatic endometriosis (n=102) and healthy females (n=93). Detection of +331G/A and PROGENS polymorphisms was performed using PCR-restriction fragment length polymorphism (RFLP) analysis. Expression of PR-A and PR-B protein was assessed with immunohistochemistry. The allelic frequency of the polymorphic allele +331A was lower in women with endometriosis ($p<0.01$) and adenomyosis ($p<0.02$) compared with healthy females. The frequency of the PROGENS polymorphism did not differ between the groups. The mean staining index (SI) for PR-B in endometriotic epithelium was higher in the presence of the +331A polymorphic allele (n=2) ($p<0.001$) compared to +331G/G individuals. The PROGENS polymorphism did not affect the SI for PR-A and PR-B.

Conclusions

The presence of the PR gene polymorphic allele +331A is associated with a reduced risk of deep infiltrating endometriosis and adenomyosis. The PROGENS polymorphism does not seem to modify the risk of deep infiltrating endometriosis.

Introduction

Endometriosis is one of the most commonly encountered benign problems in gynecology. Women with symptomatic endometriosis suffer from dysmenorrhea, dyspareunia, chronic pelvic pain and/or subfertility. Past research indicates that a combination of immunologic, genetic, anatomic and hormonal factors contribute to a woman's susceptibility to the development of endometriosis. Regarding the pathogenesis of this disease Sampson's retrograde transplantation theory is the most widely accepted, which implies that there is reflux of viable endometrial fragments through the fallopian tubes during menstruation, with subsequent adhesion, implantation and growth on and into the peritoneum (1). It has been argued that all women with patent tubes develop asymptomatic peritoneal endometriosis to a certain extent during their lifetime (2). However, Sampson's theory does not explain why only in a minority of women retrograde menstruation results in the development of symptomatic endometriosis. Endometriosis has long been recognized as having heritable tendencies and is considered a polygenic disease with a multifactorial etiology (3-6). Genetic association studies have been performed for a number of candidate genes potentially involved in the pathogenesis of endometriosis (7-11). However, so far results have been inconsistent.

A gene of specific interest within this context is the progesterone receptor (PR) gene. Progesterone is a potent antagonist of estrogen-induced proliferation in the endometrium and as such may play a pivotal role in the pathogenesis of endometriosis. The actions of progesterone are mediated by two functionally distinct receptor isoforms, PR-A and PR-B, which are expressed from a single gene as a result of transcription from two alternative promoters (12). The PR-A and PR-B isoforms are ligand-dependent members of the nuclear receptor family that are structurally identical, except for an additional 164 amino acids at the amino terminal of PR-B (13, 14). This region encodes a transactivation function that is specific to PR-B and is required to specify target genes that can be activated by PR-B but not PR-A (15). The ability of either PR-A or PR-B to activate transcription of progesterone target genes may be important in the pathogenesis of endometriosis. PR-B functions as a stronger transcriptional activator of progesterone target genes, whereas PR-A is a transcriptionally inactive dominant repressor of steroid hormone transcription activity that is thought to decrease the response to estrogen (16). Furthermore, the responses of ligand-activated PR-A and PR-B strongly depend on the cellular context. Hence, the anti-proliferative effects of progesterone depend on a tight regulation of the PR-A and PR-B isoform balance. Therefore, the hypothesis was made that DNA polymorphisms altering the activity of the protein or altering the PR-A to PR-B ratio may lead to unopposed estrogen action and therefore to an increased risk of developing endometriosis.

The PROINS polymorphism of the progesterone receptor gene has been shown to be associated with endometriosis in previous studies (17, 18). This polymorphism consists of a 320 bp PV/HS -1 Alu insertion in intron G, a silent point mutation in exon 5 (H770H), and

a single amino acid change in exon 4 (V660L) (19). The functional consequences of the PROGINS polymorphism are not yet fully understood and are still a subject of investigation.

The +331G/A polymorphism is located in the promoter region of the PR-gene and gives rise to an increased synthesis of PR-B by generating an additional TATA-box, thereby altering the PR-A to PR-B ratio (20). This polymorphism has not been investigated before with respect to the risk of developing endometriosis.

Deep infiltrating endometriosis, for example in the rectovaginal space, histologically closely resembles adenomyosis in the uterine wall. This might imply that the pathogenesis of these two entities is similar. In order to ascertain if the presence of the PROGINS polymorphism or the +331G/A polymorphism contributes to the risk of developing these subtypes of endometriosis, we investigated the prevalence of these polymorphisms in women with deep infiltrating endometriosis and women with adenomyosis in the uterine wall. The prevalence of these polymorphisms in two groups of women without symptomatic endometriosis served as control. In addition to this, we determined the expression levels of PR-A and PR-B in endometriotic lesions by immunohistochemistry in relation to the presence of either polymorphism.

Materials and Methods

Patients and tissue specimens

Patients with a surgical and histological diagnosis of deep infiltrating endometriosis who were operated between 1998 and 2004 in the University Hospital of Maastricht (n=72) were included in the study. Deep infiltrating endometriosis was defined as the presence of one or more deep infiltrating lesions in the bowel wall, vaginal wall, bladder wall and/or rectovaginal septum. Patients with adenomyosis in the uterine wall (n=40) were selected from the Dutch National Pathology Database (PALGA). The first control group consisted of gynecological patients without symptoms of endometriosis who underwent a hysterectomy for benign indications such as prolapse, pelvic pain or menstrual cycle disorders (gynecological controls, n=102). Patients with uterine fibroids and postmenopausal women were excluded from this group. A second group of controls was taken from a DNA-control panel derived from female subjects who had been referred to the Clinical Genetics department for molecular testing of various disease genes. Only subjects that were tested negative for these diseases were included in the control panel, as they can be regarded as healthy, random controls (population controls, n=93). Both patients and controls originated from the same geographic region of the Netherlands.

DNA isolation

In patients with deep infiltrating endometriosis, adenomyosis in the uterine wall and the gynecological control group the analyses were performed on DNA isolated from paraffin-embedded archival tissue. After reviewing all paraffin-embedded tissue specimens for the presence of endometriotic and/or adenomyotic lesions, five consecutive sections of 20 µm were cut from each tissue sample. Genomic DNA isolation was performed using proteinase K (Qiagen, Hilde, Germany) digestion in combination with the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA), following the recommendations of the manufacturer.

The analyses of the population control group were performed on DNA isolated from blood. Genomic DNA was isolated from 8 ml whole blood (EDTA tubes) using the Wizard Genomic DNA Purification Kit (Promega, Leiden, the Netherlands) following the manufacturer's protocol.

PROGINS and +331G/A detection

Detection of both polymorphisms was performed using restriction fragment length polymorphism (RFLP) analysis. From the patients with deep infiltrating endometriosis, adenomyosis and the gynecological controls only paraffin-embedded archival tissue was at our disposal, which is known to yield lower amounts of DNA of inferior quality compared to DNA isolated from blood or fresh tissue. Therefore, a nested PCR approach, previously described and validated by Pijnenborg and coworkers, was used to obtain a higher sensitivity (21). We were able to successfully genotype 65/72 patients with deep infiltrating endometriosis, 38/40 patients with adenomyosis and 101/102 gynecological controls for the PROGINS polymorphism. For the +331G/A polymorphism, 65/72 patients with deep infiltrating endometriosis, 39/40 patients with adenomyosis and 91/102 of gynecological controls were genotyped successfully. In the population control group DNA isolated from blood was used for analyses, and we were able to successfully genotype all 93 individuals in this group for both polymorphisms.

For detection of the PROGINS polymorphism, samples were analyzed for the V660L mutation in exon 4. Upon digestion with *BrsI* (isoschizomer *BrsSI*, Promega) the 138 bp PCR fragment was cut in three bands (95, 23 and 20 bp) in case of the V660 allele (indicated as T1 allele), or only two bands (115 and 23 bp) in case of the PROGINS allele (indicated as T2 allele). Electrophoresis was performed with the use of a 3% agarose gel stained with Gelstar (Cambrex, ME, USA) prior to casting for visualisation of the PCR products (Figure 1). A similar approach was used for detection of the +331G/A polymorphism. The 206 bp fragment amplified by PCR was digested by *NlaIV* (isoschizomer *BspLI*, Fermentas) generating two bands (149 and 57 bp) in case of the most common allele (+331G) or one band (206 bp) in case of the polymorphic allele (+331A). The obtained digestion products were loaded on a 2% agarose gel stained with Gelstar (Cambrex, ME, USA) for electrophoresis (Figure 2). All PCRs were performed in a volume of

20 µl containing 100 ng of genomic DNA, 50 nM of each primer, 0.1 mM of each dNTP, 1 unit of Taq DNA polymerase (Qiagen, Hilden, Germany), and 1x buffer with 2.5 mM MgCl₂. In all four groups only women of reproductive age were included (range: 20-50 years old). All samples were analyzed in duplo for both polymorphisms. In case of a discrepancy between the first and second result, the analysis was repeated. If the result remained inconclusive, the sample was omitted from the analysis.

Immunohistochemistry

The PgR636 mouse monoclonal antibody (DAKO, Copenhagen, Denmark) was used to detect PR-A and PR-B, the B30 mouse monoclonal antibody (Santa Cruz Biotechnology, California, USA) was used for specific detection of PR-B. Paraffin embedded lesions of patients with deep infiltrating endometriosis or adenomyosis in the uterine wall were sectioned (5µm) and fixed on Starfrost adhesive slides (Klinipath, Duiven, the Netherlands). Sections were deparaffinized in xylene and rehydrated in alcohol series prior to blocking endogenous peroxidase activity by incubation with 3% hydrogen peroxide/methanol for 20 min. After rinsing three times with phosphate-buffered saline (PBS, pH 7.2), antigen retrieval was performed (Tris-EDTA, pH 9.0; microwave treatment: 20 min at 650W). Sections were cooled down to room temperature and washed again three times in PBS followed by incubation with the primary antibody (room temperature, 2 h). After three PBS rinses, sections were exposed to the secondary antibody (Envision rabbit anti-mouse, ChemMateTM detection kit, DAKO, Copenhagen, Denmark) for 30 min at room temperature. Antibody binding was visualized using 3, 3'-diaminobenzidine. Sections were counterstained mildly with hematoxylin, dehydrated and mounted in Entellan for light microscopy.

Figure 1 Restriction fragment length polymorphism analysis of the V660L mutation in exon 4 of the PROGINs complex of aberrations. wt: patient not carrying the PROGINs polymorphic allele (T1/T1 individual); he: heterozygous PROGINs carrier (T1/T2 individual) ; ho: homozygous PROGINs carrier (T2/T2 individual).

Figure 2 Restriction fragment length polymorphism analysis of the +331G/A polymorphism. wt: patient not carrying the +331A polymorphic allele (+331G/G individual). he: heterozygous +331A carrier (+331G/A individual); ho: homozygous +331A carrier (+331A/A individual)

Evaluation of immunostaining in endometriotic tissues

The percentage of stained endometrial stromal and epithelial cells (0, 0-10, 10-50, >50%) and the intensity of staining (absent, weak, moderate, strong) were determined (0, 1, 2 or 3 for each variable) for the entire lesion. A staining index (SI, ranging from 0 to 9) was calculated for both the epithelial and stromal fraction of endometriotic lesions by multiplying categorized parameters. Two different observers (P.G.G. and K.v.K.) performed evaluation of immunostaining in a blinded fashion and independently. Both observers scored the sections once. The mean of these two observations was used for analyses.

Statistical analysis

Hardy-Weinberg equilibrium for the two polymorphisms was assessed in each group by comparing the Pearson's goodness of fit with a chi square distribution with one degree of freedom (Institute of Human Genetics, Munich, Germany: <http://www.ihg.gsf.de/>). Allele and genotype frequencies were compared between groups using Fisher's exact test. The odds ratio (OR) was used to measure the strength of the association between the frequencies of allele and genotype and deep infiltrating endometriosis.

The difference in PR-A and PR-B expression between patients with the +331G/A or PROGINS polymorphism and women without either polymorphism was determined by using a two-sample t-test with separate variance estimates. Calculations were done using SPSS software (version 11.5). All *P* values were two-tailed and 95% CI's were calculated. *P*<0.05 was considered to be statistically significant.

Results

PROGINS

Results are summarized in Table 1 and 2. The allele T1 homozygote (T1/T1) was the dominating genotype in the group of women with deep infiltrating endometriosis (66.2%) (Table 1), in the group of women with adenomyosis in the uterine wall (71.1%), the gynecological control group (73.3%) and the population control group (71.0%). The overall frequency of the PROGINS complex of genomic alterations (T1/T2, heterozygote + T2/T2, homozygote) was 33.8% in the group of women with deep infiltrating endometriosis, 28.9% in women with adenomyosis in the uterine wall, 26.7% in the gynecological control group and 29.0% in the population control group. The T2/T2 homozygous PROGINS genotype was found in 4.2% of women with severe endometriosis, 5.3% of women with adenomyosis, 1% of women in the gynecological control group and 3.2% of women in the population control group. None of the observed differences were statistically significant.

Table 1 Genotype and allele frequencies of the PROGINS polymorphism in women with deep infiltrating endometriosis, women with adenomyosis and controls

PROGINS	Deep infiltrating endometriosis (n=65)	Adenomyosis (n=38)	Gynecological controls (n=101)	Population controls (n=93)
Genotypes				
T1/T1	46 (66.2%)	27 (71.1%)	74 (73.3%)	66 (71.0%)
T1/T2	21 (29.6%)	9 (23.7%)	26 (25.7%)	24 (25.8%)
T2/T2	3 (4.2%)	2 (5.3%)	1 (1.0%)	3 (3.2%)
T1/T2 + T2/T2	24 (33.8%)	11 (28.9%)	27 (26.7%)	27 (29.0%)
Alleles				
T1	113 (81.0%)	63 (82.9%)	174 (86.1%)	156 (83.9%)
T2	27 (19.0%)	13 (17.1%)	28 (13.9%)	30 (16.1%)

n = number of patients that could be genotyped successfully

Table 2 Presence of polymorphic allele PROGINS and risk of deep infiltrating endometriosis and adenomyosis

	OR	95% CI	P-value
Deep infiltrating endometriosis versus population controls	1.24	0.70-2.21	0.47
Deep infiltrating endometriosis versus gynecological controls	1.49	0.83-2.65	0.18
Adenomyosis versus population controls	1.07	0.53-2.19	0.86
Adenomyosis versus gynecological controls	1.28	0.63-2.63	0.57

OR: odds ratio; 95% CI: 95% confidence interval

+331 G/A

Results are summarized in Tables 3 and 4. The percentage of women carrying the +331A polymorphic allele was lower in patients with deep infiltrating endometriosis (3.0%) and adenomyosis (2.6%) than in the gynecological control group (8.8%) and the population control group (18.3%). The presence of the polymorphic allele +331A was associated with a significant reduction of the risk of having the disease phenotype in women with deep infiltrating endometriosis (OR 0.22; 95% CI 0.06-0.77, $P<0.01$) and adenomyosis (OR 0.12, 95% CI 0.02-0.95, $P<0.02$) compared with women from the population control group. Compared with gynecological controls, statistical significance was not reached. There was no significant difference in the distribution of the +331G/A polymorphism between women with adenomyosis and women with deep infiltrating endometriosis. There was one homozygous +331G/A carrier in the group of women with deep infiltrating endometriosis and one in the population control group. In the other two groups no homozygous +331G/A carriers could be observed.

Table 3 Genotype and allele frequencies of the +331G/A polymorphism in women with deep infiltrating endometriosis, women with adenomyosis and controls

+331 G/A	Deep infiltrating endometriosis (n=65)	Adenomyosis (n=38)	Gynecological controls (n=91)	Population controls (n=93)
Genotypes				
+331G/G	63 (96.9%)	37 (94.6%)	83 (91.2%)	76 (81.7%)
+331G/A	1 (1.5%) ³	1 (2.6%) ¹	8 (8.8%)	16 (17.2%)
+331A/A	1 (1.5%)	0 (0.0%)	0 (0.0%)	1 (1.1%)
+331G/A + A/A	2 (3.0%)	1 (2.6%)	8 (8.8%)	17 (18.3%)
Alleles				
+331G	127 (97.7%)	75 (98.7%)	174 (95.6%)	168 (90.3%)
+331A	3 (2.3%) ²	1 (1.3%) ¹	8 (4.4%)	18 (9.7%)

n = number of patients that could be genotyped successfully. 1 = $P<0.05$ compared to population controls

2 = $P<0.01$ compared to population controls; 3 = $P<0.001$ compared to population controls

Table 4 Presence of polymorphic allele +331A and risk of deep infiltrating endometriosis and adenomyosis

	OR	95% CI	P-value
Deep infiltrating endometriosis versus population controls	0.22	0.06-0.77	0.01
Deep infiltrating endometriosis versus gynecological controls	0.51	0.13-1.98	0.37
Adenomyosis versus population controls	0.12	0.02-0.95	0.02
Adenomyosis versus gynecological controls	0.29	0.04-2.36	0.29

OR: odds ratio; 95% CI: 95% confidence interval

Immunohistochemistry

Immunohistochemical staining could be performed on 61/69 lesions from women without either polymorphism, 31/35 lesions of women with the PROGINS polymorphism and 2/3 lesions from women with the +331A polymorphic allele. Photographs of representative PR-A/B and PR-B staining are shown in Figures 3A-C. The overall level of PR-B expression was low in all three groups. In most endometriotic lesions, no staining was observed. Strong staining with the PgR636 antibody is therefore related to the presence of PR-A.

PR-A/B

Results are summarized in Figure 4A. The presence of the PROGINS polymorphic allele (T2) did not affect the SI for PR-A/B in endometrial epithelium (5.6 ± 2.7 in PROGINS carriers versus 5.5 ± 3.1 in T1/T1 individuals) or stroma (5.4 ± 2.2 in PROGINS carriers versus 5.3 ± 2.5 in T1/T1 individuals). The expression of PR-A/B in the stromal fraction of endometriotic lesions in patients carrying the +331A polymorphic allele ($SI=6.4 \pm 3.0$) did not differ significantly from that in +331G/G patients ($SI=5.3 \pm 2.5$). There also was no significant difference in the expression of PR-A/B in the epithelium of endometriotic lesions between patients carrying the +331A allele ($SI=3.8 \pm 3.8$) and +331G/G patients ($SI=5.5 \pm 3.1$).

Figure 3A Representative examples of heterogeneous expression of PR-A/B and PR-B protein in endometriotic lesions in non-carriers (patients carrying neither the +331A nor the PROGINS polymorphic allele). (For color figure see page 151)

Figure 3B Representative examples of heterogeneous expression of PR-A/B and PR-B protein in endometriotic lesions in patients carrying the PROGINS polymorphic allele. The left panel shows weak expression of PR-A/B and PR-B, whereas the right panel shows strong expression. (For color figure see page 151)

Figure 3C Representative examples of strong staining for PR-A/B and PR-B in endometriotic lesions of patients carrying the +331A polymorphic allele. (For color figure see page 152)

PR-B

Results are summarized in Figure 4B. There was no difference in mean SI for PR-B in endometriotic epithelium ($SI=0.2 \pm 0.4$ versus 0.2 ± 0.5) or stroma ($SI=0.4 \pm 0.6$ versus 0.3 ± 0.5) between PROGINS-carriers (T1/T2 + T2/T2) and T1/T1 individuals. The mean SI for PR-B in endometriotic epithelium was significantly higher in the presence of the +331A polymorphic allele ($SI=2.0 \pm 0$ in +331A carriers versus 0.3 ± 0.5 in +331G/G patients, $P<0.001$). There was no significant difference in mean staining index for PR-B in

endometriotic stroma between +331A carriers and +331G/G individuals ($SI=1.5 \pm 0.7$ vs. 0.3 ± 0.5).

Figure 4 Staining indices (SI) for **(A)** PR-A/B and **(B)** PR-B protein expression in endometriotic lesions. +331G/A: patients carrying the +331A polymorphic allele (+331G/A, heterozygous and +331A/A, homozygous). PROGINS: patients carrying the PROGINS polymorphic allele (T1/T2, heterozygous and T2/T2, homozygous). Non-carriers: patients carrying neither the +331A nor the PROGINS polymorphic allele (+331G/G and T1/T1 individuals). Results are shown for glandular epithelium (white bars) and stroma (striped bars) separately, and are presented as mean $\pm$ SD. * $p < 0.001$ compared with non-carriers.

Discussion

Endometriosis is an estrogen-dependent disease and an adequate response to progesterone in the endometrium is required to antagonize estrogen-dependent proliferation. Structurally and functionally intact PRs are essential to mediate the antiproliferative effects of progesterone on the growth of endometriotic tissue (22).

Aberrations in the PR gene, such as DNA polymorphisms, may cause a diminished response to physiological levels of progesterone and may therefore lead to an increased risk of developing endometriosis. However, in the present study we show that the PROGINS polymorphism was not associated with an increased risk of deep infiltrating endometriosis or adenomyosis in the uterine wall. Interestingly, the presence of the +331A polymorphic allele was shown to be associated with a statistically significant

decrease in risk of deep infiltrating disease and adenomyosis in the uterine wall, which is suggestive of a protective effect. Furthermore, the frequencies of both polymorphisms in women with adenomyosis in the uterine wall did not differ significantly from the frequencies observed in women with deep infiltrating endometriosis. This supports the contention that, based on histological criteria, endometriotic nodules of the rectovaginal septum and adenomyosis of the uterine wall are similar entities (23).

To this date, three studies have been carried out on the relationship between the presence of PROGINS and the risk of developing endometriosis. In two studies a statistically significant association between PROGINS and endometriosis was found (17, 18). However, findings in the third study agreed with our results and failed to provide support for an association between the PROGINS allele and endometriosis (24). Even though we detected a higher frequency of the PROGINS T2 allele in women with deep infiltrating endometriosis compared to gynecological and population controls, statistical significance was not reached. It is not surprising that conflicting results are published in genetic association studies. Ioannidis and coworkers have shown that only 16% of genetic associations identified were subsequently replicated with formal statistical significance (25). He also showed that in a large number of genetic associations a differential magnitude of effect was noted in large versus small studies. Moreover, results from first studies frequently differed significantly from the results of subsequent research, to the extent that large studies and subsequent research suggested weak associations or no association at all, compared with strong associations proposed by smaller studies and first research.

The relative expression of PR-A and PR-B is considered to be of great importance for an adequate response to progesterone and, as a result, for normal reproductive tissue functioning. Eutopic endometrium was shown to express both PR-A and PR-B isoforms, whereas in simultaneously biopsied peritoneal endometriotic tissue from the same patient PR-A, but not PR-B was detectable (26). The absence of PR-B conceivably leads to a perturbation of the PR-A to PR-B ratio in endometriotic implants, rendering this tissue less responsive to the anti-proliferative effects of progesterone.

In previous research, the PROGINS polymorphism was shown to increase transcriptional activity of the progesterone receptor, due to increased stability and increased half-life of both PR-A and PR-B isoforms (27). Whether this has any functional consequences for the progesterone receptor remains unclear. Expression levels of PR-A and PR-B are probably not affected, as we did not see a consistent increase in the expression of PR-A and PR-B in endometriotic tissues from women bearing the PROGINS allele.

Past research has shown that the +331A polymorphic allele creates a unique transcription start site by generating an additional TATA-box. As a consequence, transcription of the progesterone receptor gene is increased, favoring the production of PR-B (20). We were able to show that the presence of the +331G/A heterozygous genotype is associated with a significant decrease in the risk of developing deep infiltrating endometriosis. Like PROGINS, the +331G/A polymorphism of the progesterone receptor gene has also been

studied with respect to the risk of developing several gynecological malignancies. In one study, the presence of the +331A polymorphic allele was associated with a reduced risk of endometrioid and clear cell ovarian cancers (28). There was also preliminary evidence of a protective effect of this polymorphism against endometriosis in controls. However, in contrast to the current study, the diagnosis of endometriosis in the former study was self-reported and was not supported by medical records or surgical and/or histological evidence of the disease.

One could hypothesize that overproduction of PR-B may correct the imbalance between PR-A and PR-B in endometriotic tissue. In this manner, the development and growth of endometriotic implants may be prevented to the extent that they become symptomatic. This point of view is supported by the fact that we find higher expression levels of PR-B in endometriotic epithelium of +331A carriers. However, we need to emphasize the fact that, although this result is statistically significant, the group of +331A carriers in this study that could be analyzed immunohistochemically in this study consists of only two patients. It may therefore not be possible to draw any definitive conclusions on the effect of the presence of the +331G/A polymorphic allele on the expression levels of PR-A and PR-B. Another limitation to our findings is the fact that it was not possible to satisfyingly evaluate the results for deep infiltrating endometriosis and adenomyosis separately, as in both groups only one +331A carrier could be observed. The association we found needs to be confirmed in a larger number of patients. Future research should be aimed at gaining more insight in the mechanism through which the +331 G/A polymorphism exerts its protective effect against the development of endometriosis.

Strikingly, the decrease in risk of developing deep invasive endometriosis as a result of carriership of the +331G/A polymorphism only reached statistical significance when women with deep infiltrating endometriosis were compared with population controls. When compared with gynecological controls, this was not the case. A possible explanation for this finding is the fact that a control group consisting of gynecological patients is probably biased with respect to the prevalence of (undiagnosed) endometriosis. Although the women in our gynecological control group had not reported symptoms of endometriosis, a hysterectomy was performed as a result of gynecological complaints such as pelvic pain or irregular or heavy menstrual bleeding. Given the fact that endometriosis can cause similar complaints but may not always be recognized as such, it may explain why the prevalence of underreported and undiagnosed endometriosis is higher in this group compared with the general population.

In conclusion, we have shown that the presence of the PR gene polymorphic allele +331A reduces the risk of deep infiltrating endometriosis and adenomyosis in the uterine wall compared to the +331G/G genotype. In contrast, the PROGINS polymorphism does not seem to modify the risk of developing deep infiltrating endometriosis or adenomyosis. Moreover, our findings indicate that a general population-based control group is more suitable for this field of research than a probably biased control group consisting of gynecological patients.

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Chapter 5

DNA methyltransferases and methyl-CpG binding domain proteins in human endometrium and endometriosis

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Abstract

Objective

To determine (1) expression levels of both DNA methyltransferases (DNMTs) and methyl-CpG binding domain proteins (MBDs) in human endometrium throughout the menstrual cycle and in eutopic and ectopic endometrium of endometriosis patients and (2) hormone responsiveness of DNMT and MBD expression in explant cultures of proliferative phase endometrium

Design

In vitro study

Setting

Academic Medical Center

Patients

Premenopausal women with and without endometriosis

Interventions: Explant cultures of proliferative phase endometrium were treated with vehicle, 17 β -estradiol (E2), or a combination of E2 and progesterone (E2+P) for 24 hours

Main outcome measures

Expression levels of DNMT1, DNMT2, DNMT3B and MBD1, MBD2 and MeCP2 using real-time quantitative PCR

Results

Expression levels of DNMT1 and MBD2 were significantly higher in secretory phase endometrium compared to proliferative endometrium and menstrual endometrium. In explant cultures, treatment with E2+P resulted in significant upregulation of DNMT1 and MBD2. Expression levels of several DNMTs and MBDs were significantly lower in endometriotic lesions compared to eutopic endometrium of women with endometriosis and disease-free controls.

Conclusions

These findings suggests a role for DNMTs and MBDs in the growth and differentiation of the human endometrium and support the notion that endometriosis may be an epigenetic disease.

Introduction

Endometriosis is a benign gynecological disorder that is characterized by the presence of endometrial glands and stroma at ectopic sites outside the uterine cavity. Its exact pathogenesis remains enigmatic. Recent reports support a role for epigenetic processes such as DNA methylation in the pathogenesis of endometriosis (1-4). Epigenetics refers to the study of heritable changes in gene expression that occur *without* changes in the underlying DNA sequence (5). Several types of epigenetic regulatory mechanisms exist, including DNA methylation, histone modifications and posttranscriptional gene regulation by micro-RNAs (miRNAs) (6, 7). The most studied epigenetic alteration is DNA methylation, which refers to the addition of a methyl group to the 5' position of cytosines that precede a guanine in the DNA sequence, the CpG dinucleotide. Unmethylated CpGs are clustered in CpG islands, and are often associated with transcriptional start sites in promoter regions. DNA methylation is accompanied by post-translational histone modifications that modulate DNA function, regulating chromatin structure and determining the transcriptional state of the associated DNA. Nowadays it is widely recognized that promoter hypermethylation is associated with condensed chromatin and silencing of gene expression. The importance of this phenomenon has been extensively demonstrated in the context of cancer research, where certain tumor-suppressor genes were shown to be inactivated by promoter hypermethylation.

The mammalian DNA methylation machinery is composed of two components. DNA methyltransferases (DNMTs) are enzymes that catalyze the addition of methyl groups to CpG dinucleotides. DNMT1 is regarded as the major enzyme responsible for maintaining existing DNA methylation patterns during replication. It is located at the replication fork and methylates newly synthesized DNA strands directly after each replication round. DNMT3A and DNMT3B are thought of as *de novo* methyltransferases, which are involved in the post-replicative methylation of previously unmethylated DNA. Finally, DNMT2 is an enzyme with strong sequence similarities with 5-methylcytosine methyltransferases of both prokaryotes and eukaryotes but was shown not to methylate DNA (8).

The crosstalk between DNA methylation and histone modifications is established by a family of proteins that contain a methyl-CpG binding domain, commonly known as MBD proteins (MBDs). On the basis of several studies (9, 10), a model has been proposed for DNA methylation-mediated transcriptional silencing. According to this model, MBDS specifically recognize and subsequently bind to methylated CpGs and recruit histone deacetylases (HDACs), either directly or via a corepressor complex. The HDAC removes acetyl groups from the lysines on the NH₂-terminal histone tails, resulting in chromatin condensation, inaccessibility of transcription factors, and transcriptional inactivation. The process of gene silencing therefore requires the combined action of both DNMTs and MBDS.

To date, five MBDs have been cloned: MeCP2, MBD1, MBD2, MBD3, and MBD4. Of these, MeCP2, MBD1, and MBD2 have been shown to recruit HDAC and function as transcriptional repressors (11).

A significant correlation was demonstrated between DNA methylation and the expression of PR in normal endometrium, suggesting that DNA methylation may be involved in steroid hormone dependent endometrial growth and differentiation during the menstrual cycle (12). In addition, overexpression of steroidogenic factor-1 (2) and estrogen receptor 2 (1) was shown to be the result of demethylation of the gene promoters.

To date, two studies addressing DNMT expression in endometrium (13) and endometriosis (14) have been performed. However, these studies have focused on DNMT expression solely, and, so far, no studies reporting MBD expression in these tissues are known. Therefore, the aim of this study is to investigate the expression levels of both DNMTs and MBDs in (i) normal endometrium throughout the menstrual cycle and (ii) eutopic and ectopic endometrium of women with endometriosis. A second aim is to investigate hormone responsiveness of DNMTs and MBD expression. The presence of DNMT and MBD proteins in these tissues was assessed by immunohistochemistry.

Materials and Methods

Tissues

As described in more detail below, tissues were collected in two different clinics, the Maastricht University Medical Center (MUMC) in Maastricht, the Netherlands and the Leuven Fertility Clinic (LFC) in Leuven, Belgium.

Tissues collected in the MUMC

Endometrial tissue was collected from all phases of the menstrual cycle from 23 women with regular menstrual cycles. These women underwent surgery for benign indications other than endometriosis in the University Hospital of Maastricht. The tissue was collected from hysterectomy specimens or by pipelle biopsies during laparoscopy (Pipelle catheter, Unimar Inc., Prodimed, Neuilly-Enthelle, France). All women signed an informed consent, as required by the protocol approved by the Medical Ethical Committee of the University Hospital Maastricht. Of the twenty-three biopsies collected, 3 were collected in the menstrual phase, 8 were collected in the proliferative phase and another 12 were collected in the secretory phase of the menstrual cycle. Each biopsy was divided in three parts: the first part was immediately frozen in liquid nitrogen and stored at -80°C until used for research in this study, the second part was fixed in 10% buffered formalin for histology. Of the proliferative phase tissue samples, a third part was kept apart and directly used for endometrial explant cultures (see below). The endometrium was dated

according to clinical information with respect to the start of the last menstrual period, which was reconfirmed by histological examination of the tissue (15).

Explant cultures

Part of the proliferative phase endometrium from 8 biopsies collected in the proliferative phase of the menstrual cycle was used for endometrial explant cultures as described earlier (16). The tissue was cultured for 24 hours in the presence of vehicle (0.1% ethanol) or steroid hormones (estradiol, E2, 1 nM; progesterone, P4, 1 nM; E2 plus P4, 1 nM each). At the end of the experiment, the explants were collected in lysis buffer (SV Total RNA Isolation Kit, Promega, Madison, WI, USA) and stored at -80°C until RNA isolation. The steroid hormones were gifts from Organon pharmaceuticals (Oss, The Netherlands).

Tissues collected in the LFC

Patient material used for the present investigation has been described before (17). In short, tissue was obtained during the proliferative phase of the menstrual cycle from 34 women, including 20 women with laparoscopically confirmed absence of endometriosis 14 women with both laparoscopically and histologically confirmed stage III-IV endometriosis [American Society for Reproductive Medicine classification (18)]. Both eutopic and ectopic tissue was harvested. All patients had a regular menstrual cycle and were documented not to be on any steroid medication within six months before surgery.

RNA isolation and cDNA synthesis

Total cellular RNA from the cultured explants and uncultured endometrium was extracted using the SV total RNA isolation kit (Promega) according to the manufacturer's protocol. RNA from the tissue samples collected in the Leuven Fertility Clinic was isolated using the TRIzol reagent method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quantity and quality was assessed with a spectrophotometer at 260 and 280 nm wavelengths. For the production of the complementary DNA (cDNA) the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) was used.

Real-time PCR

The TaqMan Gene Expression Assays for DNMT1 (Hs00154749_m1), DNMT2 (Hs00189402_m1), DNMT3B (Hs00171876_m1), MBD1 (Hs00242770_m1), MBD2 (Hs00187506_m1), and MeCP2 (Hs00172845_m1), β -actin (Hs99999903_m1), and cyclophilin A (Hs99999904_m1) were purchased from Applied Biosystems (Foster City, CA, USA). Human cyclophilin A was used as housekeeping gene to normalize the differences in the amount of cDNA added to each PCR reaction. To check for the amount of endometrial tissue present in eutopic and ectopic endometrial biopsies, the expression level of 17 β -

hydroxysteroid dehydrogenase 4 (17 β HSD4), a gene previously shown to be expressed at equal levels in the eutopic endometrium and endometriotic tissue, was measured (17).

Immunohistochemistry

A summary of the primary and secondary antibodies, antigen retrieval steps and incubation conditions used is given in Table 1. Antibody binding was visualized using the Envision rabbit anti-mouse (for mouse monoclonal antibodies), or anti-goat (for goat polyclonal antibodies) ChemMate™ detection kits (DAKO, Copenhagen, Denmark) followed by incubation with 3, 3'-diaminobenzidine. Negative control slides for the monoclonal antibodies were incubated with mouse IgGs of the same class and same dilution as the primary antibodies. Negative control slides for the goat polyclonal antibodies were incubated with goat IgG at the same dilution as the polyclonal antibodies.

Statistical tests

Statistical tests were carried out using the Statistical Package for the Social Sciences 16.0 (SPSS Inc., Chicago, IL) statistical analysis package. To evaluate whether expression levels varied significantly throughout the menstrual cycle, the non-parametric unpaired Mann–Whitney *U* test was used to test for differences between these expression levels versus the expression level in the M phase. The non-parametric Wilcoxon signed-rank test was used to test for differences between steroid-treated explants and controls at a confidence level of 95%.

Table 1 Primary antibodies and conditions used for immunohistochemistry of eutopic endometrium and endometriosis lesions

Antibody	Species	Catalog number	IgG class	Dilution	Manufacturer	Incubation	Antigen retrieval
Dnmt 1	Goat	sc-10222	IgG1	1:750	Santa Cruz Biotechnology, Santa Cruz, CA, USA	2 hours room temperature	Tris-EDTA (pH 9.0)
MBD-2	Goat	sc-9397	Not specified	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Overnight 4°C	Tris-EDTA (pH 9.0)
5-MeCyd	Mouse	MCA2201	IgG1	1: 100	Abd Serotec, Oxford, UK	2 hours room temperature	Citrate (pH 6.0)

Results

DNMT and MBD expression throughout the menstrual cycle

The relative expression level of DNMT1 was significantly higher in secretory endometrium compared with proliferative ($P=0.02$) and menstrual endometrium ($P=0.009$). Expression

levels of DNMT2 and DNMT3B were not significantly different between the cycle phases. Significantly higher expression levels of MBD2 were observed in secretory endometrium compared with proliferative ($P=0.004$) and menstrual ($P=0.018$) endometrium. There were no statistically significant differences in MBD1 and MeCP2 expression throughout the menstrual cycle (Figure 1). Immunoreactivity for DNMT1 (Figure 4A), MBD2 (Figure 4B) and 5-methylcytosine (Figure 4C) was observed in nuclei of epithelial and stromal cells of both proliferative and secretory endometrium.

Figure 1 Expression levels of DNMT1, DNMT2, DNMT3B, MBD1, MBD2 and MeCP2 throughout the menstrual cycle.

Expression levels of DNMTs and MBDs in explant cultures

Because transcript levels of DNMT1 and MBD2 were shown to be upregulated significantly in the secretory phase of the menstrual cycle, hormonal regulation of these genes was hypothesized. Therefore, the effect of cultivation with estrogen (E2) alone or estrogen in combination with progesterone (E2+P) was assessed in explant cultures of proliferative endometrium. Expression of DNMT1 was significantly upregulated by treatment with E2 + P ($P=0.021$), but not by treatment with E2 alone ($P=0.086$). The same phenomenon was

observed for MBD2: expression levels were shown to be significantly higher when endometrium was cultured with E2+P ($P=0.038$), but not after cultivation with E2 alone (Figure 2).

Figure 3 Expression levels of DNMT1, DNMT2, DNMT3B, MBD1, MBD2 and MeCP2 in eutopic endometrium of controls (normal), eutopic endometrium of endometriosis patients (eutopic) and ectopic endometrium of endometriosis patients (ectopic).

Expression levels of DNMTs and MBDs in matched samples of eutopic and ectopic endometrium

DNMT1 showed significantly lower expression levels in eutopic ($P=0.007$) and ectopic ($P=0.001$) endometrium of endometriosis patients compared with eutopic endometrium of controls. Expression levels of DNMT3B did not differ between eutopic endometrium of cases and controls, but were significantly lower in ectopic endometrium compared with eutopic endometrium of cases ($P=0.008$) and eutopic endometrium of controls ($P=0.002$). The expression of DNMT2 did not vary between the groups. MeCP2 expression levels were significantly higher in eutopic endometrium of controls compared with eutopic endometrium of cases ($P=0.02$), but when they were compared with ectopic endometrium no statistically significant difference could be observed.

Figure 2 Expression levels of DNMT1 and MBD2 in endometrial explant cultures after cultivation for 24 h with estrogen (E2) or a combination of estrogen and progesterone (E2+P). Data are presented as means $\pm$ SEM. * $P < 0.05$ versus control (uncultured) endometrium.

Expression levels of MBD1 on the other hand did not vary between eutopic endometrium of controls and cases, but were significantly lower in ectopic endometrium compared to eutopic endometrium of cases ($P = 0.017$) and eutopic endometrium of controls ($P < 0.0001$). Finally, significantly lower expression levels of MBD2 were observed in eutopic ($P = 0.001$) and ectopic ($P = 0.0002$) endometrium of cases compared to eutopic endometrium of controls (Figure 3). Immunoreactivity for DNMT1 (Figure 4A), MBD2 (Figure 4B) and 5-methylcytosine (Figure 4C) was observed in epithelial and stromal cells of both eutopic and ectopic endometrium of patients.

Discussion

DNMT1 and MBD2 expression vary throughout the menstrual cycle

In the present study, we observed cyclical variations in the expression of DNMT1 and MBD2, with significantly higher expression levels of both enzymes in secretory phase endometrium compared with proliferative and menstrual phase endometrium. Furthermore, we showed that the expression of these genes is regulated by steroid hormones.

Strikingly, our findings contradict the report of Yamagata and coworkers, who found significantly reduced levels of DNMT1, 3A and 3B in the secretory phase of the menstrual cycle. Incubation of cultured proliferative phase endometrial stromal cells in the presence of estradiol and progesterone resulted in significant downregulation of DNMT3A and 3B.

Figure 4 Representative photographs of DNMT1 **(A)** and MBD2 **(B)** protein immunostainings in normal endometrium throughout the menstrual cycle (proliferative phase and secretory phase) and in eutopic and ectopic endometrium of endometriosis patients.

Figure 4 Representative photographs of 5-methyl cytosine **(C)** immunostainings in normal endometrium throughout the menstrual cycle (proliferative phase and secretory phase) and in eutopic and ectopic endometrium of endometriosis patients.

The expression of DNMT1 was not influenced under these conditions (13). A possible explanation for this discrepancy could be that the results of these authors were obtained in cultures of isolated endometrial stromal cells, whereas in the present study endometrial tissue cultures were used. Short-term explant cultures have several features which are relevant for understanding the complex intra- and inter-cellular mechanisms of tissue responsiveness to estrogen (16) and progesterone (18). Essential to the short-term explant cultures is the preservation of the tissue architecture, which is a prerequisite for the interactions between the epithelium, stroma, and extracellular matrix. It has been well documented that stromal-epithelial interactions are indispensable for the hormonal response of epithelial cells of the female reproductive tract (19) and that steroid sex hormones regulate epithelial proliferation via paracrine mechanisms requiring the appropriate receptor in the stroma. In culture systems using either isolated endometrial stromal or epithelial cells, the tissue architecture, and thus, stromal-epithelial interaction, is lost. Therefore, results obtained in these culture systems probably do not accurately reflect the *in vivo* response of human endometrium to steroid hormones.

DNMT1 is considered the maintenance DNMT, since its primary function is to copy an already established DNA methylation pattern after replication. Based on this functional characteristic, it would be expected that DNMT1 expression levels are highest in the proliferative phase of the menstrual cycle. However, in the present study expression levels of DNMT1 in the secretory phase were shown to exceed those in the proliferative phase. Interestingly, it has been shown that DNMT1-depleted isolated ES cells remain viable and proliferative, but die upon induction of differentiation (20-22). Also, targeted deletion of DNMT1 in mouse embryo's proved to be lethal, with embryonic death occurring during or after gastrulation when the pluripotent embryonic stem cells begin to differentiate. These findings indicate a role for DNMT1 not only in proliferating cells, but also in the subsequent differentiation of cells.

The second protein that was shown to be significantly upregulated in the secretory phase is MBD2. There is no evidence for an essential embryonic function for any mammalian methyl-CpG binding protein (23), illustrated by the fact that MBD2 and MeCP2 null mice are viable and fertile and display no overt phenotype (24, 25). However, there is evidence for overlapping functions in differentiation and specification of neural stem cells in culture and in postnatal animals (23). Furthermore, it was shown that MeCP2 and MBD2 expression increases during (terminal) differentiation (26), which is in line with our finding of elevated expression levels of MBD2 in secretory phase endometrium.

Reduced expression levels of DNMTs and MBDs in endometriosis

In the present study we show that expression levels of DNMT1 and MBD2 are significantly lower in eutopic and ectopic endometrium of endometriosis patients compared to eutopic endometrium of controls, whereas MeCP2 expression is lower in eutopic than in ectopic endometrium of endometriosis patients. Finally, expression levels of DNMT3B and MBD1

are lower in ectopic endometrium compared to eutopic endometrium of both endometriosis patients and controls.

Our findings do not confirm the observations of Wu and coworkers who found increased expression levels of DNMT1, DNMT3A and DNMT3B in ectopic endometrium as compared to disease free control subjects and eutopic endometrium of women with endometriosis (14). Possible explanations for this discrepancy may be related to the heterogeneous patient population used by Wu and coworkers (menstrual, proliferative and secretory phase endometrium versus only proliferative phase endometrium in the present study), and the use of isolated endometrial epithelial cells rather than tissue cultures.

As stated above, DNMT1, MBD2 and MeCP2 appear to be indispensable for tissue-specific gene expression and differentiation. The findings obtained in the present study may signify that both eutopic and ectopic endometrium of endometriosis patients have a reduced capacity for differentiation and hence, decidualization and implantation. Circumstantial evidence in support of this notion has been provided by numerous studies: aberrant expression of progesterone receptor (PR) in endometriotic tissues (27-29), the absence of the estradiol inactivating enzyme 17- β -HSD2 in eutopic and ectopic endometrium of endometriosis patients in the secretory phase of the cycle (30) and altered expression of HOXA10 –a gene that is critical for endometrial decidualization and implantation- in eutopic and ectopic endometrium of endometriosis patients (31, 32). Furthermore, levels of the decidualization markers prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) are reduced in supernatants from endometrial stromal cells derived from all three types of ectopic lesions and from eutopic endometrium of women with endometriosis (33). Results obtained in a recent study by Aghajanova and coworkers also suggest resistance to full decidualization of endometrial stromal fibroblasts in women with endometriosis (34). Taking all this into account, it is tempting to speculate that reduced capacity for differentiation of endometriotic cell lines and endometrial cells from women with endometriosis may influence the capacity for proliferation and survival of these cells in the ectopic environment maintaining the ectopic endometrial tissue. Moreover, the changes in the expression of DNMTs and MBDs in endometriotic tissue seem to have functional consequences in the endometrium and endometriotic tissue as illustrated by the overexpression of SF-1 (2) and ER2 (1) genes as a result of promoter demethylation, whereas the expression of PR-B is suppressed due to hypermethylation of the promoter (3).

In conclusion, we have shown that expression levels of DNMT1 and MBD2 vary throughout the menstrual cycle. Their anticipated dependence on steroid hormones was confirmed in explant cultures prepared from proliferative phase endometrium tissue. The significant reduction in the expression of various DNMTs and MBDs in ectopic endometrium of endometriosis patients may explain why altered gene promoter methylation has been observed in endometriotic lesions. The fact that these changes in methylation status are also associated with changes in phenotype, i.e. gene expression, supports the notion that endometriosis may be an epigenetic disease.

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Chapter 6

Is the extreme dysregulation of genes in eutopic endometrium of endometriosis patients the result of aberrant gene promoter methylation?

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Submitted

Abstract

Background

Recent reports have provided support for a role of epigenetic mechanisms in the pathogenesis of endometriosis. One of the basic regulatory epigenetic mechanisms is DNA methylation, occurring in genes that harbor a CpG rich region (CpG island) in their promoter sequence. DNA methylation is commonly associated with complete silencing of a gene. A recent microarray-based study has shown that several genes are more than 100 fold up- or downregulated in the eutopic endometrium of women with endometriosis compared to eutopic endometrium of healthy controls in the window of implantation. We hypothesize that the striking severity of up- or downregulation is the result of gene promoter methylation.

Methods

Genes that were >100 fold up- or downregulated in eutopic endometrium of women with endometriosis were selected based on biological plausibility and investigated for the presence of significant CpG islands in the promoter region, yielding 10 genes of interest: PRIM2A, TGF β 3 EFNB1, TAF6L, SHB, ITGA2, BENE, Rap1GAP, OGG1 and PRB. Genomic DNA and RNA was isolated from eutopic endometrium samples of women with rAFS stage III/IV endometriosis (n=14) and controls (n=14). All samples were obtained in the proliferative phase of the menstrual cycle. Quantitative real-time PCR was used to investigate mRNA expression levels of the genes of interest in eutopic endometrium samples of endometriosis patients (n=6) and controls (n=6). Promoter methylation status was assessed by methylation specific PCR (MSP).

Results

Both mRNA expression levels and methylation status of the investigated regions of the promoter CpG island in all genes investigated were not significantly different between the groups.

Conclusion

Based on the findings in the present study it is not possible to draw any definitive conclusions on the regulation of the investigated genes by (promoter) methylation. We recommend that our hypothesis may be retested in a larger cohort of eutopic endometrium samples, with the use of modern, sensitive, core-region specific techniques for the detection of DNA methylation.

Introduction

Endometriosis is a benign, estrogen-dependent disease of the female genital tract that affects millions of women worldwide. Its exact pathogenesis remains enigmatic, but it is thought that genetic factors contribute to the initiation and progression of the disease. One of the approaches in the search for genes causatively related to the pathogenesis of endometriosis is gene expression profiling. Numerous microarray studies have revealed differential expression of varying sets of genes in endometriosis patients. Comparisons have been made between different individuals (eutopic endometrium of affected versus unaffected women) (1-4) or between different tissues from the same individual (eutopic endometrium versus ectopic endometrium) (5-10). The process of regulation of gene expression is highly complex and can take place at many different levels, such as transcription, post transcriptional splicing of RNA, translation and post translational modification of proteins. Epigenetic regulatory processes such as DNA methylation are able to modulate gene expression by interfering with chromatin configuration and subsequent accessibility of the chromatin for transcription factors. The DNA methylation pattern acts as a cell type-specific memory of transcriptional activity and provides a broad range of gene expression rates, varying from high-level expression to complete silencing. Typically, hypermethylation of CpG islands located in the 5'promoter region of genes is associated with gene silencing, whereas hypomethylated regions are associated with enhanced gene activity.

Recent reports support a role for DNA methylation in the pathogenesis of endometriosis. The expression of certain genes such as estrogen receptor-2 (11), steroidogenic factor-1 (12), aromatase (13), HOXA10 (14), progesterone receptor B (15) and E-cadherin (16) were shown to be regulated by promoter hyper- or hypomethylation in eutopic endometrium and ectopic lesions of women with endometriosis.

In 2003, a microarray study by Kao et al. demonstrated that a number of genes were severely (>100 fold) up- or downregulated in eutopic endometrium of women with endometriosis compared to eutopic endometrium of their disease-free counterparts (3) in the window of implantation. Given the striking extent of respective up- or downregulation, we hypothesized that the expression of the genes that have CpG islands in their promoter region is regulated by promoter methylation in these tissues.

Materials and Methods

Patients and tissue specimens

Tissues were collected in the Leuven Fertility Clinic (LFC) in Leuven Belgium. This set of patients consisted of 34 women that underwent laparoscopic surgery for infertility with or without pelvic pain in the University Hospital of Leuven. Of these women, 20 had no

laparoscopically visible endometriosis, whereas 14 women had both laparoscopically (rAFS stage III-IV) and histologically confirmed endometriosis. Eutopic endometrial tissue was obtained from all women by endometrial biopsy after hysteroscopy and before laparoscopy using a sterile Pipelle cannula (Pipelle de Cornier, Paris, France). All biopsies were taken in the proliferative phase of the menstrual cycle. All patients had signed an informed consent, as required by the protocol approved by the Medical Ethical Committee of the University Hospital Leuven. All women had a regular menstrual cycle and were documented not to be on any kind of steroid medication within six months before surgery. Of these samples, 28 were used for analysis, 14 eutopic endometrium samples of endometriosis patients, and 14 eutopic endometrium samples of disease-free controls.

RNA isolation

RNA was isolated using the TRIzol reagent method (Invitrogen, Calsbad, CA, USA) according to the manufacturer's protocol. RNA quantity and quality was assessed with a spectrophotometer at 260 and 280 nm wavelengths. For the production of the complementary DNA (cDNA) the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) was used.

DNA isolation

Genomic DNA was extracted from endometrium pipelle biopsies by using a proteinase K (Qiagen, Hilden, Germany) digestion followed by DNA isolation using the Puregene DNA Isolation Kit (Gentra Systems, MN, USA).

Selection of genes

Genes that were >100 fold up- or downregulated in eutopic endometrium of women with endometriosis in the study by Kao et al. were selected based on biological plausibility (involvement in processes such as apoptosis, angiogenesis, cell-cycle regulation, inflammation and adhesion). The promoter sequence of these genes was recovered (Ensembl) and investigated for the presence of significant CpG islands in the promoter region with the aid of appropriate software (CpG Plot, <http://www.ebi.ac.uk/Tools/emboss/cpgplot/>). Progesterone receptor B (PRB) was added to the list of genes, because PRB has already been shown to be regulated by promoter hypermethylation in ectopic lesions of endometriosis (15). A summary of the genes investigated and their involvement in different pathways is given in Table 1.

Table 1 Genes investigated in the present study and their involvement in different pathways *based on results by Kao et al.

Gene	GenBank ID	Full description	Function	Up/down in endometriosis*
PRIM2A	X74331	DNA primase subunit p58	DNA replication	Up
TGFβ3	M60556	Transforming growth factor β3	Signal transduction, cell growth, differentiation, apoptosis	Up
EFNB1	L37361	Ephrin-B1	Cell adhesion, differentiation	Up
TAF6L	AF069735	PCAF-associated factor 65 α	RNA polymerase	Down
SHB	X75342	Src homology 2 domain containing adaptor protein B	Signal transduction, apoptosis	Down
BENE	U17077	T-cell differentiation protein-like	Differentiation, apical trafficking	Down
ITGA2	X17033	Integrin α2 subunit	Cell adhesion	Down
Rap1GAP	M64788	GTPase activating protein	Signal transduction	Down
OGG1	Y11731	DNA glycosylase	DNA repair	Down
PRB	M15716	Progesterone receptor B	Differentiation	Down

DNA modification and methylation specific PCR (MSP)

Sodium bisulphite modification of DNA samples was carried out using reagents provided in the EZ DNA methylation Kit (Zymo Research, CA, USA). In this reaction, unmethylated cytosines are converted to uracil whereas those that are methylated (5-methylcytosine) are resistant to this modification and remain as cytosine. Subsequently, DNA subjected to bisulphite modification was amplified by PCR with primers specific for methylated and unmethylated DNA (methylation specific PCR). The methylation status of the promoter regions of selected genes was determined by a nested MSP approach, in order to increase the sensitivity of detecting allelic hypermethylation at targeted sequences. Step one of the nested MSP was carried out with primer sets (sense and anti-sense) flanking the CpG rich promoter regions of the 10 respective targeted genes in separate reactions. PCR products of step one were diluted 1:1000 and subjected to the second step that incorporated one set of primers for each gene [labeled unmethylated (U) and methylated (M)] that were designed to recognize bisulfite-induced modifications of unmethylated (U) and methylated (M) cytosines. Methylation was defined as the presence of (1) only an M band or (2) the presence of both U+M bands. If the result was inconclusive, it was omitted from the analysis. Primer sets and PCR conditions for both steps of the nested MSP are listed in Table 2. DNA isolated from normal peripheral lymphocytes from healthy individuals served as a negative methylation control (NL). Normal lymphocyte DNA was treated *in vitro* with SssI methyltransferase (New England Biolabs, Ipswich, MA) to create completely methylated DNA at all CpG-rich regions. *In vitro* methylated DNA (IVD) served as the positive methylation control.

Table 2 Primer sets and PCR conditions for methylation specific DNA (U: unmethylated DNA; M: methylated DNA)

Primer set	Sense primer	Antisense primer	Amp size (bp)	Annealing temperature (°C)	No of PCR cycles
PRIM2A flank	5'-AATTGGTGTGGTGGTTTGA-3'	5'-AAAAAATAAAACACTAAAAATCAC-3'	100	56	35
PRIM2A U	5'-TGGTGTGGTGGTTTGTATGTTT-3'	5'-AACACTAAAAATCACACAAACATACCA-3'	86	60	35
PRIM2A M	5'-GGTGTGGTGGTTTGTACGTTTC-3'	5'-AAAATCACGCGAACGTACCG-3'	78	60	35
TGFβ3 flank	5'-YGTAGGTTGGGAGTTTTTTTG-3'	5'-TCTCCCTCCTAATCCCCC-3'	129	56	35
TGFβ3 U	5'-ATGGTTGTTTTATTGGGTTTGT-3'	5'-CCCCCAATCCCAAAACCA-3'	84	66	35
TGFβ3 M	5'-TCGTTTTTATCGGTTTCGC-3'	5'-CCCGATCCGAAACCG-3'	78	66	35
EFNB1 flank	5'-TAGTGGGAGGTTTGTGAGGT-3'	5'-CRAACCAATCCCATTAAATTAC-3'	135	56	35
EFNB1 U	5'-GAGGTTTGTATTGGTTGTAGATTTT-3'	5'-AAACCTCAACCAATAACCCA-3'	91	66	35
EFNB1 M	5'-GTTCTGATTGGTCGTAGATTTTC-3'	5'-CCTCGACCGAATAACCG-3'	85	66	35
TAF6L flank	5'-CTGTAGTAAGAGAGGTTGTTT-3'	5'-ACTCACACTCATTCCCTCTCCC-3'	193	57	35
TAF6L U	5'-GGGAATTTTGTGTTATGTGATTGT-3'	5'-CCCAATACAACCAATCAACAAACA-3'	124	60	28
TAF6L M	5'-AATTTTACGCTTACGTATCGTC-3'	5'-CAATACAACCAATCGACGAACG-3'	119	60	28
SHB flank	5'-GTGTGTAGGAGYGGAGAGGTG-3'	5'-CAACACCRCCCATCC-3'	102	56	35
SHB U	5'-GGAGAGGTGTGTGTTATTTTGGTTTT-3'	5'-CAAACTAACCCCAACCCACA-3'	72	66	35
SHB M	5'-GAGGTGCGTTATTTTCGTTTC-3'	5'-CCTAACCCGGAACCGCG-3'	65	66	35
BENE flank	5'-GGTAGGAGGTGATATTGTAGAGT-3'	5'-AAACAGAAAAAATAATAAAAAACAA-3'	158	54	35
BENE U	5'-GAGGTGATATTGATAGGTTGTGTGT-3'	5'-AATAAAAAACCAACACACCCCA-3'	140	60	28
BENE M	5'-GATATTTGATAGTTGCGCGC-3'	5'-AAACAACGCGACCCCGC-3'	129	60	28
ITGA2 flank	5'-GGATAGTTTTTGTAGAGTGTGAGGTTT-3'	5'-CCAACAGAACCCCAAAAAAC-3'	234	57	35
ITGA2 U	5'-TTTAGAGTGTGAGGTTTTGTATTTTTT-3'	5'-CAAAACCCCAAAAAACCAACA-3'	222	50	30
ITGA2 M	5'-TTAGAGTGTGAGGTTTTCGTATTTTC-3'	5'-CAACAACGACAACGACGCG-3'	120	50	30
Rap1GAP flank	5'-GGTAGGGYGGGGGTTTTT-3'	5'-CRCCRCTACAACCTCTACTCAAATAC-3'	164	56	35
Rap1GAP U	5'-GGGGGTTTTTTTGTGTTTGTGT-3'	5'-CTACAACCTCACTCAAAATACAACCAACA-3'	150	66	35
Rap1GAP M	5'-GGTTTTTCGTCGTTTTCGC-3'	5'-AACTCTACTCAAAATACGACGCG-3'	144	66	35
OGG1 flank	5'-GGGGYGTTTAGATGGAATT-3'	5'-CRCCAATAAAAAATCAAAATAAAC-3'	141	56	35
OGG1 U	5'-GTTTAGATGGAATTTGTAGTGTGTTT-3'	5'-ATAAACAAACCATTTCTAAACACTCA-3'	119	64	35
OGG1 M	5'-TAGATGGAATTCGTTAGCGTTTC-3'	5'-GAACCGTTCTAAAACGCTCG-3'	110	64	35
PRB flank	5'-TGACTGTCGCCCGCAGTACG-3'	5'-CGGCAATTTAGTGACACGCG-3'	200	57	40
PRB U	5'-TGATTGTTGTTGTAGTATG-3'	5'-CAACAATTTAATAACACACA-3'	200	45	40
PRB M	5'-TGATTGTCGTTTCGTAGTACG-3'	5'-CGACAATTTAATAACACGCG-3'	200	51	40

Real-time PCR

Real-time quantitative PCR was performed with the ABI Taqman Gene Expression system (purchased from Applied Biosystems, Foster City, CA) for PRIM2A, TGFβ3, EFNB1, TAF6L, SHB, BENE, ITGA2, Rap1GAP, OGG1 and PRB. Twelve eutopic endometrium samples were used for these experiments: six from women with laparoscopically and histologically confirmed endometriosis and six from disease-free controls. Samples from the same 12 women had also been used for analysis of promoter methylation status by MSP. Primers were designed with the aid of appropriate software (<http://primerdepot.nci.nih.gov/>) and are listed in Table 3. Human β actin was used as house-keeping gene to normalize the differences in the amount of cDNA added to each PCR reaction. Relative quantification of mRNA species was performed using the comparative threshold cycles (CT) method. In brief, CT was used to determine the mRNA level normalized to the average mRNA level in endometrium biopsies. Thus, mRNA levels were expressed as an n-fold difference. For each sample, the gene CT value was normalized using the formula: $\Delta CT = CT_{\text{gene}} - CT_{\beta \text{ actin}}$. To determine relative expression levels, the following formula was used: $\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{calibrator}}$. This value was used to plot the gene expression employing the formula $2^{-\Delta\Delta CT}$.

Table 3 Primer sets used for quantitative real-time (RT) PCR

Gene	Sense	Antisense
PRIM2A	5'-GAGGTGATTGAGCAGAGGCT-3'	5'-AGCCAACTGTCCAAGGC-3'
TGFβ3	5'-CACATTGAAGCGGAAACCT-3'	5'-AAATTCGACATGATCCAGGG-3'
EFNB1	5'-GCAGATGATGTCCAGCTTGT-3'	5'-AAGAACCTGGAGCCCGTATC-3'
TAF6L	5'-CTCTTCTCGCTCTGACATGG-3'	5'-GAGTGTGAGCTGGTGAGTGG-3'
SHB	5'-CTTGGCATCAAAGGGATCTG-3'	5'-GAAACTGCTCAACAAGTGCG-3'
BENE	5'-GGGGTATACTATGTGGGTGGC-3'	5'-TGTTCTCACCATCCCTTTC-3'
ITGA2	5'-TCACTTGAAGGACCGGAAAA-3'	5'-CTGGTGTAGCGCTCAGTCA-3'
Rap1GAP	5'-ACAGGTCTAGTGCCTGAGGG-3'	5'-CCCTTGGGGTGGACAAG-3'
OGG1	5'-GAGATGAGCCTCCACCTCTG-3'	5'-CTTTTGGACCTCGGCTCATC-3'

Statistical tests

Statistical tests were carried out using the Statistical Package for the Social Sciences 16.0 (SPSS Inc., Chicago, IL) statistical analysis package. Promoter methylation frequencies were compared between the groups with the Fisher's exact test. To evaluate whether expression levels of the investigated genes varied significantly between the two groups, the non-parametric unpaired Mann-Whitney U-test was used. All p-values were two-tailed and a p level <0.05 was considered to be statistically significant.

Results

MSP in eutopic endometrium of women with endometriosis versus controls

Gene promoter methylation in PRIM2A was present in 17% (2/12) of eutopic endometrium samples of controls and completely absent in eutopic endometrium of endometriosis patients (0/12), but the difference was not statistically significant ($p=0.48$). There were no statistically significant differences in methylation status of TGFβ3, EPNB1, TAF6L, ITGA2, BENE, Rap1GAP, OGG1 and PRB between eutopic endometrium of endometriosis patients and eutopic endometrium of controls. Gene promoter methylation in TGFβ3, TAF6L, ITGA2, Rap1GAP and PRB was completely absent in both eutopic endometrium of women with endometriosis and controls. In the case of EPNB1, 100% of samples in both groups were methylated (U+M) (12/12 and 12/12, respectively). BENE showed promoter methylation in 27% (3/11) of eutopic endometrium of controls and in 31% (4/13) of eutopic endometrium of endometriosis patients (Table 4). MSP for SHB was considered inconclusive, because of an unexplained absence of signal (U and M bands) in all samples, except in the positive (IVD) and negative (NL) methylation control.

Table 4 Prevalence of promoter methylation (M) in eutopic endometrium of endometriosis patients and controls

Gene	M endometriosis (%)	M controls (%)
PRIM2A	0/12 (0%)	2/12 (17%)
TGFβ3	0/12 (0%)	0/12 (0%)
EFNB1	12/12 (100%)	12/12 (100%)
TAF6L	0/5 (0%)	0/12 (0%)
BENE	3/11 (27%)	4/13 (31%)
ITGA2	0/13 (0%)	0/14 (0%)
Rap1GAP	0/12 (0%)	0/12 (0%)
OGG1	0/12 (0%)	0/12 (0%)
PRB	0/10 (0%)	0/11 (0%)

Expression levels of investigated genes in eutopic endometrium of endometriosis patients and controls

Significantly higher expression levels of PRIM2A were observed in eutopic endometrium of endometriosis patients compared to eutopic endometrium of controls ($p=0.008$) (Figure 1). Expression levels of TGFβ3, EPNB1, TAF6L, SHB, BENE, ITGA2, Rap1GAP and PRB were not significantly different between the groups, although a trend could be observed for higher expression levels of Rap1GAP and PRB in eutopic endometrium of endometriosis patients compared to controls (Figure 1). RT-PCR for OGG1 was considered inconclusive because of a high level of background noise caused by primer dimers, which distorted the melting curve.

Discussion

Epigenetic regulatory mechanisms such as DNA methylation have been implicated in the pathogenesis of endometriosis, illustrated by the fact that several genes involved in estrogen metabolism were shown to be regulated by promoter methylation in eutopic and ectopic endometrium of endometriosis patients. Hypermethylation of the CpG rich promoter region is commonly associated with complete silencing of gene expression. To date, several microarray studies have revealed differential expression of genes in the eutopic endometrium of endometriosis patients compared to disease-free controls (1-4). Based on the striking extent of up- or downregulation (>100 fold) of certain genes harboring a significant CpG island in their promoter region (PRIM2A, TGFβ3, EFNB1, TAF6L, SHB, ITGA2, BENE, Rap1GAP, OGG1 and PRB) in one of these studies (3), we hypothesized that the expression of these genes may be regulated by promoter methylation.

Figure 1 Expression levels of investigated genes in eutopic endometrium of disease-free controls (left) and eutopic endometrium of endometriosis patients (right). * $P < 0.05$ compared to eutopic endometrium of controls.

In the present study, we observed no statistically significant differences in methylation status of the investigated regions of the promoter CpG island between women with endometriosis compared to controls. In the following section, the interpretation of these data and the methodology used in the present study will be critically discussed within the context of recent and novel insights on the subject of DNA methylation and its exact relation to gene expression.

Not long ago, a rather strict correlation between promoter hypermethylation and gene silencing was assumed. This dogma is currently being revisited, as it has become clear that specific regions within a CpG island (“core regions”) are crucial for the regulation of gene expression (17). These core regions are often situated around the transcription start site (TSS) within a CpG island, but can also be observed upstream or downstream of the TSS. Evidence is accumulating that transcriptional silencing depends on the methylation status of these gene-specific core regions within the CpG island, irrespective of the methylation

status of adjacent CpG sites. A core region may be limited to merely a few CpG dinucleotides, as illustrated by a recent study demonstrating that methylation of only one specific CpG dinucleotide at the border of the CpG island is associated with transcriptional silencing of the TTP gene in liver cancer (18). Therefore, the exact location of hypermethylation seems to be the most important factor in determining whether or not a gene is silenced.

Furthermore, regulation of expression by hypermethylation of CpG dinucleotides is not necessarily restricted to the promoter area. It can also be observed in intra- and intergenic regions that may function as alternative promoters (19), in enhancers (i.e. *cis*-regulatory DNA sequences that increase transcription independent of their orientation and distance relative to the TSS) (20, 21), and in bidirectional promoters (22, 23). Strikingly, hypermethylation can even regulate expression in promoters without a classical CpG island (i.e. promoters with low CpG density) (24). Moreover, hypermethylation throughout the gene locus by means of long-range interactions with the promoter region has been described within the context of phenomena such as chromatin looping (25) and long-range epigenetic silencing (LRES, i.e. the concept that clusters of genes can be coordinately repressed by epigenetic mechanisms) (26-29).

Because of these newly identified location-related complexities of gene expression regulation by aberrant DNA methylation, data generated by MSP analysis need to be interpreted with extreme caution. The results of bisulfite-based analyses such as MSP are highly dependent on primer design. The most important limitation of MSP in this respect resides in the fact that only 2 to 4 CpG dinucleotides per primer are assessed by this technique. In order to investigate the methylation status of the core region, MSP primers would need to perfectly cover the core region of interest. In the MSP analysis used in the present study, primers were designed without knowledge of the (location of) core regions for the genes investigated. Therefore, it can be concluded that no differences in methylation exist for the CpG dinucleotides in the promoter area that were covered by the primers used in the present study. However, no conclusion can be drawn on the regulation of these genes by (promoter) methylation, because the core regions crucial for the regulation of gene expression have not been analyzed. Another compromising factor is the lack of confirmation of significant differences in expression levels of the investigated genes by quantitative real-time PCR (except for PRIM2A). This could be due to the fact that no actual differences in expression levels exist, or to the fact that the sample size in the present study is too small to detect these differences.

A great variety of technologies is available for the analysis of DNA methylation, most of which are based on one of three main principles: methylation-sensitive restriction enzymes, bisulphite conversion of unmethylated cytosines and immunoprecipitation of 5-methylcytosines (30). The kind of information obtained (qualitative, quantitative, resolution etc.) highly depends on the method chosen. Therefore, it is critical to choose the approach that fits the planned research objectives best. Up until now, MSP has been one of the most widely used methods to detect CpG island methylation (31). It is simple,

sensitive (to 0.1% methylated alleles of a given CpG-rich locus) and requires small quantities of DNA, allowing for the study of paraffin-embedded or microdissected tissue samples. In retrospect however, taking the most recent insights on core regions into account, MSP is not the appropriate assay to test our hypothesis. This is mainly because of its low resolution (i.e. its ability to detect only a few CpGs inside the primers) and its inability to quantify methylation at a given locus. A method that would be more suitable for this purpose is pyrosequencing (32). Pyrosequencing provides reproducible and accurate measures of the degree of methylation at several CpGs in close proximity in combination with a high quantitative resolution. Like MSP, this method requires the coupling of bisulphite treatment of genomic DNA with PCR amplification of the target sequence, but is followed by pyrosequencing as a final step. Pyrosequencing is a sequence-by-synthesis approach (i.e. real-time) that detects luminescence (proportional to the quantity of DNA and number of nucleotides) from the release of pyrophosphate following nucleotide incorporation into the complementary strand. The incorporation of a cytosine is indicative of a methylated residue, whereas the incorporation of a thymine indicates an originally unmethylated cytosine. Thus, the degree of methylation at each CpG site is determined from the ratio of C and T. In order to test our hypothesis accurately, this approach needs to be core region- specific, implying that the choice of region analysis is extremely important in this respect.

In summary, based on the findings in the present study it is not possible to draw any definitive conclusions on the question whether the expression of the investigated genes is regulated by (promoter) methylation. We recommend that our hypothesis may be retested in a larger cohort of eutopic endometrium samples, with the use of modern, sensitive, core-region specific techniques with validation of expression levels by qRT-PCR as a first screening test.

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Chapter 7

General Discussion

In this thesis different molecular pathways were studied with regard to their potential involvement in the initiation and progression of severe endometriosis. The relevance and clinical implications of the findings obtained in these studies as well as the future perspectives are discussed in this chapter.

Fibromuscular differentiation and TGF- β signaling in deep infiltrating endometriosis

In the first part of this thesis the origin of smooth muscle (SM) like cells in deep infiltrating endometriosis and the underlying mechanism responsible for the presence of these cells was investigated. We demonstrated that the fibromuscular tissue in deep infiltrating endometriosis lesions expresses multiple markers of SM differentiation, similar to the situation encountered in pathological wound healing and fibrocontractive diseases. In a nude mouse model we showed that the expression of alpha-smooth muscle actin (ASMA) was induced in murine tissue after implantation of human endometrial fragments, suggesting that the presence of SM-like tissue in endometriosis lesions is the result of a reaction of the local environment to the presence of ectopic endometrium.

A small number of studies reported suggestive evidence in favor of the notion that SM like cells in endometriosis lesions result from SM metaplasia of endometrial stromal cells. In one study this was based on positive staining for uterine markers estrogen receptor (ER), PR and oxytocin receptor in differentiated smooth muscle cells in the periphery of endometriosis lesions (1). However, given their location remote from the lesion and their degree of differentiation it is very well possible that these cells are in fact resident smooth muscle cells that were already present before ectopic endometrium arrived at the site. In two other studies, the presence of ASMA positive cells between endometriotic stromal cells led to the assumption that these cells were of uterine origin (2, 3). This was later contradicted by a study by Hull and coworkers, using a mouse xenograft model. Fluorescent colocalization of anti-ASMA antibodies and the Hoechst nuclear stain 14 days after transplantation of human endometrial fragments into nude mice revealed that ASMA positive myofibroblasts in central locations (i.e. in the endometrial stroma) in endometriosis lesions were of murine origin, thereby confirming our conclusion that ASMA is induced in local host cells and not in human endometrial stroma (4).

It remains to be fully elucidated whether TGF- β 1 is indeed (partially) responsible for the fibromuscular reaction observed in deep infiltrating endometriosis. Hull and coworkers investigated endometrial-peritoneal interactions during endometriotic lesion establishment in mouse xenograft lesions (4) using microarrays. One of the four key pathways identified in this study was the TGF- β pathway. RNA transcripts of TGF- β , myofibroblast-associated transcripts such as ASMA as well as ECM components were present in both human and mouse transcriptomes, supporting the importance of this pathway in endometriosis lesion formation.

With respect to the source that TGF- β is derived from in endometriosis, peritoneal macrophages are likely candidates. It has been demonstrated that TGF- β 1 levels are elevated in peritoneal fluid of women with endometriosis (5) and it has been postulated that this is the result of secretion of TGF- β by activated macrophages in the peritoneal fluid. It is known that two major macrophage phenotypes exist, carrying out differential functions and eliciting divergent effects on surrounding cells and tissues (6). Classically activated macrophages (M1) promote inflammation, extracellular matrix (ECM) destruction and apoptosis, while alternatively activated macrophages (M2) demonstrate a more “permissive” phenotype, promoting ECM construction, cell proliferation and angiogenesis. The differentially activated phenotype is induced by cytokines IL4 and IL13 and is thought to play a role in fibrosis, due to the release of profibrotic factors such as interleukin (IL)-1 β , TGF- β , fibronectin and matrix metalloproteinases (7). Macrophages in the peritoneal fluid and those that actively infiltrate ectopic lesions display features of alternative activation in both endometriosis patients and mice with experimentally induced endometriosis (8). Furthermore, adoptive transfer of alternatively activated macrophages into a mouse xenograft model dramatically enhanced endometriotic lesion growth, whereas classically activated macrophages significantly reduced growth of ectopic lesions (8). This suggests that the peritoneal environment in endometriosis patients induces the differentiation of macrophage precursors toward an alternatively activated, reparatory phenotype and that the alternative activation of macrophages may be a clue to the heterogeneity in the susceptibility to endometriosis.

TGF- β : a target for drug therapy in endometriosis?

Ultimately, one of the goals of identifying factors involved in the pathogenesis of endometriosis is the development of effective therapeutic strategies, and TGF- β 1 may be a potential target in this respect. Interestingly, targeted deletion of TGF- β 1 in nude mice resulted in a significantly smaller amount of ASMA positive murine myofibroblasts in endometriosis lesions and significantly lower weight of endometriosis lesions in total compared to TGF- β 1 ^{+/+} mice (9). Furthermore, it has been demonstrated that TGF- β 1 enhances transmesothelial invasion by immortalized endometrial cells and eutopic endometrial epithelial cells of endometriosis patients *in vitro* (10) and antagonists of TGF- β 1 signaling significantly inhibited cellular invasiveness. Inhibitors of TGF- β 1 signaling components may therefore be efficacious in the treatment of endometriosis.

TGF- β regulates key mechanisms of tumor development, namely immunosuppression, metastasis, angiogenesis, and proliferation, also making it an attractive target for antitumor therapies. Currently, the majority of the TGF- β signaling inhibitors are in the preclinical stage. For example, TGFBR I inhibitors were shown to be effective in inhibiting pancreatic adenocarcinoma cell growth in a mouse xenograft model and *in vitro* (11, 12). So far, the most progress has been made with the TGF beta 2 blocking oligonucleotide trabedersen, that is now being used in clinical studies (13). Intratumoral treatment with

trabedersen in patients with high-grade glioma is currently evaluated in a randomized controlled phase III study, and phase I/II studies in advanced pancreatic carcinoma, metastatic melanoma, and metastatic colorectal carcinoma are ongoing. At present, an antibody to all three isoforms of human TGF- β (GC-1008), a small-molecule inhibitor of the TGF- β RI kinase (LY573636), and a vaccine using allogeneic tumor cells have also entered clinical cancer trials (14).

Further studies are required to elucidate the mechanistic actions of (components of) the TGF- β 1 signaling pathway and its inhibitors on endometriosis lesions before targeting of TGF- β 1 is contemplated. Moreover, when designing drugs that target TGF- β 1 signaling one should be aware of undesirable side effects that may arise due to potentially blocking tumor-suppressor activity of TGF- β (15,16). Dissection of the TGF- β 1 signaling pathway may lead to the development of improved therapeutics that target specific TGF- β 1 signaling components involved in endometriosis without affecting its tumor-suppressor activity.

Suitability of animal models

Some of the results described above should be interpreted with caution because they were obtained in animal models, most often murine models. Murine models are the most frequently used to study the establishment and evolution of endometriosis in time and characterize key (molecular) events in this dynamic process. This is due to their low cost and the possibility of (1) studying the endometrial lesions at different time intervals and (2) acquiring knowledge about the effect of different drugs or treatment. In addition, a number of genetic knock out models are available, and mice are easy to manipulate genetically. Despite these advantages, it is important to recognize that the murine model presents several important limitations. First, mice lack menstruation and consequently do not develop spontaneous endometriosis. Second, the use of mouse xenograft models (i.e. in which human endometrial tissue is used) implies the use of immunocompromized rodents such as nude mice. This hampers the investigation of an immunoregulatory component involved in the pathogenesis of this disease. Third, endometriosis lesions in mice are small and are not physiologically similar to those found in advanced stages of human disease. Human endometrial tissue transplanted into nude mice only has a limited lifespan, being unable to persist beyond 4 weeks (17) and as a result, cannot acquire characteristics of more advanced stages of endometriosis. Therefore, the extent to which the induced lesions in mice are truly representative of the disease itself, and in particular advanced staged disease such as deep infiltrating endometriosis, is debatable.

Some of the limitations named above can be overcome by the use of nonhuman primates, although the use of this model is costly and faced with ethical concerns. Of the nonhuman primates, the baboon (*Papio anubis*) is the most favorable because of its phylogenetic similarity to humans, its size and similar reproductive anatomy. Baboons are a menstruating species that develop spontaneous endometriosis with ectopic tissue

morphologically identical to humans and at similar sites (18-20). Endometriosis in baboons can also be artificially induced by injecting autologous menstrual effluent into the pelvic cavity (21, 22) mimicking the process of retrograde menstruation. In baboons, it is possible to perform repetitive laparoscopies and collect biological samples before and after the induction of disease, making this model particularly useful for studying the effect of therapeutic interventions. Although the baboon arguably constitutes the most suitable animal model for endometriosis for reasons named above, it presents with a limitation similar to the murine models: minimal to moderate disease is the most common finding in nonhuman primates, whereas severe endometriosis is rarely encountered (23, 24). Again, this raises the question whether (artificially induced or spontaneous) endometriosis in nonhuman primates reflects “real” endometriosis, i.e. advanced, symptomatic disease. This may also explain why none of the recently tested drugs that showed effectiveness in reducing the extent of endometriotic lesions in animal models in the preclinical stage has reached the clinic thus far (25-27).

It may be concluded that both rodent and nonhuman primate animal models are invaluable tools for the investigation of specific (early) events involved in the pathogenesis of endometriosis, but it is important to be aware of the relevant disadvantage concerning the extent to which findings obtained in these models can be extrapolated to severe stages of endometriosis in humans. It remains to be determined to what extent the different models are predictive for evaluating clinical efficacy of new therapeutic strategies.

Genetic aberrations in selected candidate genes

In the second part of the thesis, we investigated the possible association between the presence of single nucleotide polymorphisms (SNPs) with functional consequences at the gene or protein level in two selected candidate genes and the susceptibility for developing deep infiltrating endometriosis. We concluded that no association exists between the presence of the -509C/T polymorphism in the TGF- β 1 gene or the PROGINS polymorphism in the progesterone receptor gene and deep infiltrating endometriosis. A positive association could be demonstrated between the presence of the +331G/A polymorphism in the progesterone receptor gene and a *reduced* risk of deep infiltrating endometriosis compared to population controls.

Drawbacks of the candidate gene approach

At the time when we performed our experiments, the (hypothesis driven) candidate gene study was the mainstay of genetic investigation into the vast majority of complex diseases thought to have a genetic component, but at present substantial criticism is directed at this approach. Lack of replication of genetic associations across different populations is

the main reason for this criticism. This has also proven to be true for the results obtained in our studies, because they were not consistently replicated in subsequent studies (28-34) (Table 1). There are many reasons for the lack of reproducibility encountered in a large number of candidate gene studies. The most important of these is the fact that many candidate gene studies are statistically underpowered. Complex diseases are likely to be caused by multiple (and sometimes numerous) genes, each with an overall small contribution and relative risk. Large sample sizes (typically several thousand cases and controls) are needed in order to detect the expected small effect size of a particular variant, and most candidate gene studies conducted in the field of endometriosis have failed to reach these numbers. Furthermore, genetic associations are often inconsistent across ethnic barriers, due to different frequencies of polymorphic alleles as well as gene–gene interactions. Therefore, a genetic association, although valid in a specific ethnic population, may not be relevant for individuals of another ethnicity. Candidate gene studies in endometriosis might differ in study population and the definition of the phenotype studied. Ovarian, peritoneal and deep infiltrating endometriosis are often taken together as a group and are not treated as different disease entities with potentially different etiologies- albeit with considerable overlap. As a result, non-replication of association with a particular genetic variant might result from real biological differences between different phenotypes. Conversely, evidence of genetic risk in a homogenous population of endometriosis without subtype investigation is likely to lead to spurious associations. Moreover, problems such as confounding, different types of bias and misclassification are more likely to obscure small- to-moderate relative risks than larger relative risks.

Some of these methodological problems can (in part) be overcome by summarizing data from individual studies in the form of a meta-analysis. The only consistent association revealed by a recent meta-analysis of genetic association studies so far is a positive association between the presence of a phase II detoxifying enzyme GSTT1 polymorphism and the risk of endometriosis. There was a 29% increased risk of endometriosis in GSTT1 null deletion carriers (35). However, there was evidence of publication bias in this meta-analysis, indicating that the effect size of the GSTT1 deletion may actually be smaller or non-existent (36).

Genome wide association studies in endometriosis: a new era

The candidate gene approach is a “hypothesis driven” approach, meaning that a gene identified as a candidate is hypothesized to be involved in the risk of developing endometriosis based on biological plausibility and then examined for genetic variants, usually SNPs. However, biological pathways thought to play a role in the development of endometriosis are complex and generally involve a magnitude of genes. It has been argued that incomplete understanding of such pathways renders it likely that variants and genes of possible importance are overlooked by using hypothesis driven techniques. In

Table 1 Polymorphisms of the progesterone receptor gene and the transforming growth factor $\beta 1$ gene that have been investigated for their role in endometriosis.

Gene	Variant (dbSNP ID)	Authors	Association with susceptibility endometriosis	Cases (n)	Controls (n) [†]	General population controls (n) [‡]	Population
PGR (progesterone receptor)	PROGINS (320 bp PV/HS-I Alu insertion in intron G and 2 SNPs: rs1042838 in exon 4 and rs104839 in exon 5)	Wieser et al., 2002	+	95	107	-	Austrian
		Lattuada et al., 2004	+	131	127	-	Italian
		Treloar et al., 2005	-	980 triads*	980 triads*	980 triads*	Australian/European
		Van Kaam et al., 2007a	-	72	102	93	Dutch
		De Carvalho et al., 2007	+	121	281	-	Brazilian
		Govindan et al., 2007	-	100	108	108	Indian
		Near et al., 2011	-	348	-	5464	Australian/European/US
TGF $\beta 1$ (transforming growth factor $\beta 1$)		Costa et al., 2011	+	54	443	-	Brazilian
	+331 G/A (rs 10895068)	Treloar et al., 2005	-	980 triads*	980 triads*	980 triads*	Australian/European
		Van Kaam et al., 2007a	-	72	102	93	Dutch
		Gentilini et al., 2008	+	199	161	139	Italian
		Near et al., 2011	-	348	-	5464	Australian/European/US
	-509 C/T (rs 1800469)	Hsieh et al., 2005	+	150	159	-	Taiwanese
		Van Kaam et al., 2007b	-	72	95	93	Dutch
		Kim et al., 2010	-	485	352	-	Korean

*endometriosis case and two parents, [†]control group consisting of women with (gynecological) condition or complaints in whom endometriosis was ruled out, [‡]control group consisting of healthy women derived from general population

reaction to this, the attention has shifted to high-throughput, non-hypothesis driven strategies such as the genome wide association study (GWAS). Genome wide association studies can be thought of as a large series of candidate gene studies performed in a single experiment on an array based format that capture most, although not all, common variation in the genome. Typically, several hundred thousand to more than 1 million SNPs at a time are studied in this manner. These are evenly spread throughout the entire genome and therefore require no knowledge of genes or biological pathways involved in disease pathogenesis. This type of study has now largely superseded the candidate gene approach and genetic linkage and has proven to be successful in identifying genetic variants that contribute to other complex diseases such as type 2 diabetes and rheumatoid arthritis. The results of the first GWA studies in the field of endometriosis are now emerging. Uno and coworkers found a significant association with a SNP in the tumor suppressor gene CDKN2BAS in a cohort of Japanese women. Furthermore, a possible association was found for a SNP in the linkage disequilibrium (i.e. the non-random association of alleles at two or more loci) block of CDKN2BAS containing WNT4 (37). An even larger genome-wide association study was carried out by an Australian group, who identified a novel locus on chromosome 7p15.2 that was significantly associated with risk of endometriosis in women of European ancestry (38). The identified SNP is located in an intergenic region upstream of the plausible candidate genes NFE2L3 and HOXA10. Interestingly, this group confirmed the previously reported suggestive association for SNPs close to the WNT4 locus in a cohort of British, US and Australian women. WNT4 is important for development of the female reproductive tract (39), ovarian follicle development and steroidogenesis (40, 41) and therefore also a plausible candidate gene. Future research should be aimed at elucidating whether variations in these genes truly modify the risk of endometriosis, and the mechanism by which they do so.

In conclusion, considerable debate has arisen over the best strategies to use for the genetic analysis of complex traits such as endometriosis. It is unlikely that any one approach will yield all there is to find in the human genome with regard to disease susceptibility. The advent of new techniques such as GWAS has contributed enormously to the understanding of the genetics of other complex diseases and progress is just beginning to be made in the case of endometriosis. In order to be successful, sufficiently large, well phenotyped case cohorts are required, and international collaborations are essential to replicate initial findings in a second population. Although these measures will lead to increased cost and complexity of studies, only through robust and well-designed experimental procedures we will truly be able to define which pathways contribute to the disease and begin to understand the genetic risks of endometriosis.

Endometriosis, an epigenetic disease?

The third part of the thesis addresses the question whether epigenetic regulatory mechanisms, such as DNA methylation, are involved in differential expression of genes in endometriosis patients and in regulating the functional changes in the endometrium that occur throughout the menstrual cycle. Our findings demonstrate cyclical variations in expression levels of epigenetic modulators DNMT1 and MBD2, suggesting that DNA methylation may be involved in endometrial growth and differentiation. Furthermore, the expression of these genes was shown to be regulated by steroid hormones in experiments using endometrial explant cultures. Expression levels of several DNMTs, and MBDs were significantly reduced in ectopic endometrium of endometriosis patients, possibly explaining why altered promoter methylation of certain genes is observed in endometriotic lesions. Finally, we demonstrated that expression levels of a selection of genes that were shown to be markedly up- or downregulated in eutopic endometrium of endometriosis patients in a previous microarray study are not regulated by promoter methylation.

Epigenetic regulatory mechanisms in the endometrium throughout the menstrual cycle

There is little evidence that differential expression of genes in different phases of the menstrual cycle is under control of DNA methylation. At this point in time, there is more evidence that favors the notion that histone modifications are involved in this process. Modification of histones is an accepted and ubiquitous epigenetic mechanism that allows the transient condensation or relaxation of the chromatin, and therefore controls the accessibility of transcription factors to the DNA for gene transcription. Histone deacetylation, one of the best characterized histone modifications, is associated with gene silencing, whereas histone acetylation is associated with transcriptional activation (42). Histone acetylation is implicated in the vascular endothelial growth factor pathway during angiogenesis (43), and key events in the endometrium such as proliferation, differentiation and decidualization can be influenced *in vitro* by administration of histone deacetylases (HDAC) and HDAC inhibitors (44-46), suggesting a role for histone acetylation status in these processes.

The endometrium is a dynamic tissue with large numbers of genes whose expression need to be regulated in a controlled manner during the menstrual cycle, implicating that the induced changes need to be (rapidly) reversible. It has been shown that most, if not all, histone modifications are dynamic and rapidly changing. Acetylation, methylation, phosphorylation, and deimination can appear and disappear on chromatin within minutes of a stimulus arriving at the cell surface (47). It is therefore conceivable that histone modifications such as histone acetylation are indeed involved in differential expression of genes throughout the menstrual cycle. Strikingly, preliminary data by Munro and

coworkers indicate that endometrial global histone acetylation, as determined by western blotting, changed throughout the menstrual cycle and correlated well with expected transcription activity during the different phases (48), whereas expression levels of HDACs and histone acetylases (HATs) are expressed in the human endometrium without cyclical changes (49). This implies that, although it is tempting to speculate that varying expression levels of epigenetic modulators DNMTs, MBDs, HDACs and HATs correlate with DNA methylation or global acetylation status of the endometrium throughout the menstrual cycle, at this point in time there is insufficient evidence to support this notion. More research is needed to elucidate if such a correlation exists.

Correlation between gene expression and promoter methylation in endometriosis

In line with the above named speculation, our observation of reduced levels of DNMTs and MBDs in ectopic endometrium of endometriosis would be expected to lead to overall reduced levels of DNA methylation in this tissue. Research conducted on the subject so far has revealed three specific gene promoters that are hypomethylated in ectopic endometrium (50-52) and two gene promoters that are hypermethylated (53, 54). Recent genome-wide profiling of methylated promoters in endometriosis by Borghese and coworkers demonstrated that the overall methylation profile was highly similar between eutopic endometrium and ectopic endometrium from superficial peritoneal, ovarian and deep infiltrating lesions. It showed promoter regions that were consistently hypomethylated or hypermethylated in all types of endometriosis and others that were specific to one given subtype, but hypomethylation was not more prevalent in ectopic endometrium than hypermethylation (55).

In endometriosis, it is the question whether the aberrant DNA methylation observed in eutopic and ectopic endometrium is the cause or the consequence of the disease. On the one hand, aberrant DNA methylation of gene promoters can be an innate property of the eutopic endometrium, causing changes in gene expression that lead to intrinsic differences (i.e. increased proliferation and survival in the ectopic environment) that contribute to the pathogenesis of this disease. However, it has been shown that expression levels of HOXA10, a gene critical for endometrial decidualization and implantation, are reduced in eutopic endometrium after the induction of endometriosis in mice and baboons (56). This is accompanied by promoter hypermethylation of HOXA10, which is not present in disease-free animals (57). These findings suggest that epigenetic changes in eutopic endometrium may be elicited by external factors such as the presence of ectopic endometrium and therefore represent the result of the disease rather than the cause. In the case of ectopic endometrium, chronic inflammation is thought to play a role in the epigenetic modifications that are observed in this tissue. It is generally known that DNA methylation patterns are influenced by age, diet, toxins, (intra-uterine) environment, the use of medication and chronic inflammation (58, 59). It has been postulated that

repetitive retrograde menstruation elicits a chronic inflammatory response in the local peritoneal environment, leading to aberrant methylation of gene promoters in endometrium after it arrives at ectopic locations, favoring the notion that epigenetic changes arise as a result of the disease.

A short-lived dogma?

Another striking observation in the study by Borghese and coworkers (55) is that there is no evident correlation between methylation and expression level of nearby genes in ovarian endometrioma cells. This is in apparent contradiction with the current dogma that methylated CpGs are associated with repression of transcription. However, this observation is not a new one. A recent study by Rakyan and coworkers also reports a negative correlation between DNA methylation and gene expression at promoters with a high, medium or low CpG density (60). Conversely, gene-body methylation was found to positively correlate with gene expression (43, 60, 61). This may (partially) explain why we didn't observe aberrant promoter methylation in genes that were severely up- or downregulated in eutopic endometrium of endometriosis patients. A recently discovered entity, 5-hydroxymethylation of cytosines (5hmC), may prove to be of importance in explaining this phenomenon. 5hmC is particularly enriched at promoters and in intragenic regions (gene bodies) but is largely absent from non-gene regions (62). 5-hydroxymethylation peaks at transcription start sites did not correlate with gene expression levels for promoters with intermediate or high CpG content. However, the presence of 5hmC in gene bodies was more positively correlated with gene expression levels than was the presence of 5-methylcytosine (5mC), suggesting that 5hmC may be more potent than 5mC in suppressing intragenic transcription (62).

Importantly, the most commonly used technique for DNA methylation mapping, sodium bisulfite treatment of DNA, was shown to be incapable of distinguishing between 5mC and 5hmC (63), indicating that significant limitations exist for interpreting data obtained by this technique. Specific methodologies for the detection of 5hmC are currently being developed (64) allowing for a greater understanding of the temporal and spatial effects that 5hmC will have on epigenetic regulation.

In conclusion, although indications exist that DNA methylation is involved in the pathogenesis of endometriosis, we are just beginning to understand the complexity of this mechanism. The epigenome of a cell is highly dynamic, being governed by a complex interplay of genetic and environmental factors. As knowledge on the subject deepens, the vision of a strict correlation between promoter hypomethylation on the one hand, and gene up-regulation and promoter hypermethylation and gene down-regulation on the other hand appears to be a simplification and associating methylation with complex phenotypes, especially outside the field of malignancy, has been very difficult (65). However, identifying differentially methylated regions in different types of endometriosis is useful because these may help identifying pathways involved in their (separate)

pathogeneses. The establishment of specific epigenomic profiles of different types of endometriosis could be used for diagnostic purposes and for the development of new therapeutic strategies. New techniques such as genome-wide arrays for the study of interindividual variations in methylation patterns with locus-specific resolution have been added to the repertoire of investigators only very recently (66) and may prove to be valuable in this respect in the near future.

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Summary

Endometriosis is one of the most common benign gynecological conditions with an estimated prevalence of about 10% in women of reproductive age. It is defined as the presence of functional endometrial glands and stroma at ectopic locations outside the uterine cavity, most often located on the ovaries, the fossa ovarica, the uterosacral ligaments and the pouch of Douglas. Symptoms include dysmenorrhea, dyspareunia, chronic and severe pelvic pain throughout the menstrual cycle, and infertility.

Currently, Sampson's retrograde transplantation theory is the most widely accepted theory regarding the pathogenesis of endometriosis, postulating that reflux of viable endometrial cells takes place through the fallopian tubes during menstruation with subsequent adhesion, implantation and growth on and into the peritoneum and ovary (Sampson, 1927). However, retrograde menstruation is common, and small endometriotic implants are frequently encountered in asymptomatic women of reproductive age. As a consequence, there is a growing appreciation for the notion that mild peritoneal endometriosis should be considered a physiological phenomenon rather than a disease.

In 1990, deep infiltrating endometriosis was introduced as a new distinct entity. Deep infiltrating endometriosis is defined as endometrial glands and stroma infiltrating >5 mm under the peritoneal surface. Because deep infiltrating lesions show the strongest association with symptoms such as dysmenorrhea, deep dyspareunia, chronic pelvic pain and painful defecation, this type of lesion is considered a pathological condition, as opposed to superficial peritoneal endometriosis. Moreover, deep infiltrating endometriosis shows morphological similarities to adenomyosis in the uterine wall (i.e. the presence of endometrial glands and stroma inside the myometrium).

As yet, the question why endometriosis develops into a pathological condition in some but not all women has remained largely unanswered. Environmental, genetic and epigenetic factors are thought to play a role in this process. The general aim of this thesis is to gain more insight in the contribution of each of these factors to the initiation and progression of deep infiltrating endometriosis.

An outline of the clinical background and current views on the pathogenesis of endometriosis are described in **Chapter 1**. Subsequently, the molecular pathways studied in this thesis are described in more detail. Finally, the aims of the thesis are presented with an outline of the individual chapters.

The first part of the thesis addresses the role of the local environment in the establishment and maintenance of deep infiltrating endometriosis lesions. In **Chapter 2**, we (1) characterized the fibromuscular tissue present in deep infiltrating endometriosis lesions by using immunohistochemical markers of smooth muscle differentiation, (2) investigated the origin of smooth muscle cells in endometriosis lesions with the aid of a nude mouse model and (3) investigated a possible role for the cytokine transforming

growth factor- β 1 (TGF- β 1) in this process by using immunohistochemical markers of TGF- β 1 signaling.

Fibromuscular tissue surrounding human deep infiltrating endometriosis lesions contains myofibroblastic cells that express multiple markers of smooth muscle differentiation, such as alpha-smooth muscle actin (ASMA), desmin and smooth muscle myosin heavy chain (SM-MHC). This resembles the situation encountered in pathological wound healing and fibrocontractive diseases. In a nude mouse model we showed that ASMA expression is induced in the host tissue after implantation of human endometrial fragments, suggesting that the presence of smooth muscle-like tissue in endometriosis lesions is the result of a reaction of the local environment to the presence of ectopic endometrium rather than smooth muscle metaplasia of the ectopic endometrium itself. Immunohistochemical expression of TGF- β receptor 1, TGF- β receptor 2 and marker of activated TGF- β 1 signaling phospho-Smad2 was more pronounced in the endometrial component of the lesions than in the fibromuscular component, indicating that most TGF- β 1 signaling apparently occurs in the endometrial tissue and not in the local host cells. Therefore, it is not likely that TGF- β 1 signaling alone is sufficient to account for the excessive fibromuscular tissue in deep infiltrating endometriosis lesions.

In the second part of the thesis we addressed the question whether genetic variants such as single nucleotide polymorphisms (SNPs) with functional consequences in two selected candidate genes influence the susceptibility for developing deep infiltrating endometriosis. In **Chapter 3** we evaluated the possible association between the -509C/T polymorphism of the transforming growth factor- β 1 (TGF- β 1) gene and the risk of developing deep infiltrating endometriosis. We observed no statistically significant differences in the frequency of the -509C/T polymorphism between 72 deep infiltrating endometriosis patients and two separate control groups consisting of 95 gynecological patients without symptoms of endometriosis and 93 healthy females. The -509C/T polymorphism of the TGF- β 1 gene therefore does not seem to modify the risk of developing deep infiltrating endometriosis.

The possible association between two polymorphisms of the progesterone receptor (PR) gene, PROGINS and +331G/A, and deep infiltrating endometriosis was evaluated in **Chapter 4**. The frequency of the PROGINS polymorphism did not differ between the groups and therefore does not seem to modify the risk of deep infiltrating endometriosis. The frequency of the polymorphic allele +331A was lower in 72 women with deep infiltrating endometriosis (3.0%) and 40 women with adenomyosis of the uterine wall (2.6%) compared with 93 healthy females (18.3%). The presence of the polymorphic allele +331A was associated with a statistically significant reduction of the risk of having the disease phenotype in women with deep infiltrating endometriosis (OR 0.22; 95% CI 0.06-0.77) and adenomyosis (OR 0.12, 95% CI 0.02-0.95). Strikingly, the decrease in risk of developing deep invasive endometriosis only reached statistical significance when women with deep infiltrating endometriosis were compared with a general population- based

control group of healthy females, but not when compared with a control group of 102 gynecological patients without symptoms of endometriosis (frequency of polymorphic allele +331A: 8.8%). These findings indicate that a general population-based control group is more suitable for this field of research than a probably biased control group consisting of gynecological patients.

The third part of the thesis addresses the question whether epigenetic regulatory mechanisms such as DNA methylation are involved in the regulation of endometrial growth and differentiation and in differential expression of genes in endometriosis patients. In **Chapter 5**, we investigated (1) expression levels of epigenetic markers DNA methyltransferases (DNMTs) and methyl-CpG binding domain proteins (MBDs) in human endometrium throughout the menstrual cycle and in matched samples of eutopic and ectopic endometrium of endometriosis patients and (2) hormone responsiveness of DNMT and MBD expression in explant cultures of proliferative phase endometrium. Expression levels of DNMTs and MBDs were assessed by real-time quantitative PCR.

The results of these experiments showed significantly higher expression levels of DNMT1 and MBD2 in secretory phase endometrium (n=12) compared to proliferative phase endometrium (n=8) and menstrual endometrium (n=3). Expression levels of DNMT1 and MBD2 were significantly upregulated in endometrium treated with E2+P, suggesting a role for DNMTs and MBDs in cyclic growth and differentiation of the human endometrium. Finally, expression levels of several DNMTs and MBDs were significantly lower in endometriotic lesions compared to eutopic endometrium of women with endometriosis and disease-free controls. This may explain why altered gene promoter methylation has been observed in endometriotic lesions and supports the notion that endometriosis may be an epigenetic disease.

In **Chapter 6** we tested the hypothesis that severe up- or downregulation of several genes in eutopic endometrium of endometriosis patients in a previously conducted microarray study is the result of promoter methylation. Genes were selected based on biological plausibility and investigated for the presence of significant CpG islands in the promoter region. Both mRNA expression levels and methylation status of the investigated regions of the promoter CpG island in PRIM2A, TGF β 3, EFNB1, TAF6L, SHB, ITGA2, BENE, Rap1GAP, OGG1 and PRB were not significantly different between eutopic endometrium of endometriosis patients and eutopic endometrium of controls. Because of recent and novel insights in DNA methylation and the importance of so-called “core regions” for the regulation of gene expression, our findings do not permit any definitive conclusions on the regulation of the investigated genes by (promoter) methylation. We recommend retesting our hypothesis in a larger cohort of eutopic endometrium samples, with the use of modern, sensitive, core-region specific techniques for the detection of DNA methylation.

In **Chapter 7**, the most important findings were summarized and discussed within the context of recent literature. Limitations of the thesis were mentioned, and recommendations for future research were given.

Samenvatting

Endometriose is één van de meest voorkomende goedaardige gynaecologische aandoeningen en treft naar schatting ongeveer 10% van alle vrouwen in de vruchtbare leeftijd. Het wordt gedefinieerd als de aanwezigheid van functioneel endometrium, bestaande uit klierbuisjes en stroma, op ectopische locaties buiten het cavum uteri. Voorkeurslokalisaties zijn de ovaria, de fossa ovarica, de sacro-uteriene ligamenten en het cavum Douglasi. Dysmenorroe, dyspareunie, ernstige en chronische buikpijn en subfertiliteit zijn de belangrijkste symptomen.

Op dit moment is de retrograde transplantatie theorie van Sampson wereldwijd de meest geaccepteerde theorie betreffende de ontstaanswijze van endometriose. Volgens deze theorie vindt er tijdens de menstruatie reflux van vitale endometriumcellen plaats via de tubae naar de buikholte, waarna deze cellen hechten, implanteren en verder groeien op en in het peritoneum ter plaatse. Retrograde menstruatie is echter een veel voorkomend fenomeen, en kleine endometriose laesies worden vaak gezien bij asymptomatische vrouwen in de vruchtbare leeftijd. Hierdoor wordt milde peritoneale endometriose steeds vaker beschouwd als een fysiologisch verschijnsel dan als een ziekte.

In 1990 werd diep invasieve endometriose geïntroduceerd als een nieuwe, separate entiteit. Diep invasieve endometriose wordt gedefinieerd als het voorkomen van endometriale klierbuisjes en stroma >5 mm onder het peritoneumoppervlak. Omdat diep invasieve laesies het sterkst geassocieerd zijn met symptomen als dysmenorroe, diepe dyspareunie, chronische pijn in het kleine bekken en pijnlijke defaecatie wordt dit type endometriose als pathologisch beschouwd, in tegenstelling tot oppervlakkige peritoneale endometriose. Ook vertoont diep invasieve endometriose morfologisch gelijkenis met adenomyosis uteri (d.w.z. de aanwezigheid van endometriale klierbuisjes en stroma in het myometrium van de uterus).

De factoren die ervoor zorgen dat endometriose zich in de ene vrouw wel tot een pathologische entiteit ontwikkelt en in de andere niet, zijn nog grotendeels onopgehelderd. Zowel omgevingsfactoren als genetische en epigenetische factoren worden verondersteld hierbij een rol te spelen. Het doel van dit proefschrift was om meer inzicht te verkrijgen in de invloed van elk van deze drie factoren op het ontstaan en de progressie van diep invasieve endometriose.

Een overzicht van de klinische achtergrond en huidige inzichten betreffende de pathogenese van endometriose wordt gegeven in **Hoofdstuk 1**. Vervolgens worden de verschillende moleculaire pathways die onderzocht werden in dit proefschrift in detail beschreven. Tenslotte worden de onderzoeksvragen en een overzicht van de hoofdstukken in dit proefschrift vermeld.

Het eerste gedeelte van dit proefschrift beschrijft de rol van de lokale omgeving bij het ontstaan en de instandhouding van diep invasieve endometriose laesies. In **Hoofdstuk 2** werd (1) het fibromusculaire weefsel dat aanwezig is in diep invasieve endometriose

laesies gekarakteriseerd met behulp van immunohistochemische markers van gladde spiercel differentiatie, (2) de herkomst van gladde spiercellen in endometriose laesies onderzocht in de naakte muis en (3) een mogelijke rol van het cytokine transforming growth factor- β 1 (TGF- β 1) in dit proces onderzocht met behulp van immunohistochemische markers van TGF- β 1 signaling.

Het fibromusculaire weefsel dat diep invasieve endometriose laesies omgeeft bevat myofibroblasten die meerdere markers van gladde spiercel differentiatie tot expressie brengen, zoals alpha-smooth muscle actine (ASMA), desmine en smooth muscle myosin heavy chain. Dit komt overeen met de situatie bij pathologische wondgenezing en ziekten die gekarakteriseerd worden door fibrose en contractie van weefsel. In de naakte muis toonden we aan dat de expressie van ASMA wordt geïnduceerd in het gastheerweefsel na de implantatie van humane endometriumfragmenten. Dit suggereert dat de aanwezigheid van gladde spiercel-achtig weefsel in endometriose laesies het gevolg is van een reactie van de lokale omgeving op de aanwezigheid van ectopisch gelokaliseerd endometrium, en niet van gladde spiercel metaplasie van het ectopische endometrium zelf. Immunohistochemische expressie van TGF- β receptor 1, TGF- β receptor 2 en phospho-Smad2 (een marker voor actieve TGF- β 1 signaling) was aanwezig in zowel de endometriale als de fibromusculaire component van de laesies maar was meer uitgesproken in de endometriale component, wat uitwijst dat TGF- β 1 signaling hoofdzakelijk plaatsvindt in het endometrium en niet in het gastheerweefsel. Het is daarom onwaarschijnlijk dat de hoeveelheid fibromusculair weefsel in diep invasieve endometriose laesies verklaard wordt door TGF β signaling alléén.

Het tweede gedeelte van het proefschrift behandelt de vraag of genetische varianten zoals single nucleotide polymorphisms (SNPs) met functionele consequenties in twee geselecteerde kandidaatgenen de vatbaarheid voor het ontwikkelen van diep invasieve endometriose beïnvloeden. In **Hoofdstuk 3** werd de mogelijke associatie tussen het -509C/T polymorfisme van het TGF- β 1 gen en het risico op het ontwikkelen van diep invasieve endometriose onderzocht. Er waren geen statistisch significante verschillen in de frequentie van het -509C/T polymorfisme tussen 72 diep invasieve endometriose patiënten en twee separate controlegroepen bestaande uit 95 gynaecologische patiënten zonder symptomen van endometriose en 93 gezonde vrouwen. Het -509C/T polymorfisme van het TGF- β 1 gen lijkt daarom het risico op diep invasieve endometriose niet te modifieren.

De mogelijke associatie tussen twee polymorfismen in het progesteron receptor (PR) gen, PROGINS en +331G/A, en diep invasieve endometriose werd onderzocht in **Hoofdstuk 4**. De frequentie van het PROGINS polymorfisme verschilde niet tussen de groepen en lijkt daarom het risico op diep invasieve endometriose niet te beïnvloeden. De frequentie van het polymorfe allel +331A was lager in 72 vrouwen met diep invasieve endometriose (3.0%) en in 40 vrouwen met adenomyosis uteri (2.6%) vergeleken met 93 gezonde vrouwen (18.3%). De aanwezigheid van het polymorfe allel +331A was geassocieerd met

een statistisch significante reductie van het risico op het hebben van het fenotype diep invasieve endometriose (OR 0.22; 95% CI 0.06-0.77) of adenomyosis uteri (OR 0.12, 95% CI 0.02-0.95). Opmerkelijk was dat deze afname in risico op het ontwikkelen van diep invasieve endometriose alleen statistische significantie bereikte wanneer vrouwen met diep invasieve endometriose werden vergeleken met een controlegroep met gezonde vrouwen uit de algemene populatie, maar niet wanneer zij vergeleken werden met een controlegroep bestaande uit 102 gynaecologische patiënten zonder symptomen van endometriose (frequentie polymorfe allel +331A: 8.8%). Dit geeft aan dat een controlegroep die representatief is voor de algemene populatie geschikter is voor dit type onderzoek dan een controlegroep bestaande uit gynaecologische patiënten, omdat bij deze laatste groep het risico op de introductie van bias verhoogd is.

Het derde gedeelte van dit proefschrift behandelt de vraag of epigenetische mechanismen zoals DNA methylering betrokken zijn bij de regulatie van groei en differentiatie in het endometrium en bij de differentiële expressie van genen in endometriose patiënten. In **Hoofdstuk 5** onderzochten we (1) de expressie van de epigenetische markers DNA methyltransferases (DNMTs) en methyl-CpG-binding domain proteins (MBDs) in humaan endometrium gedurende de menstruele cyclus en in eutopisch en ectopisch endometrium van endometriose patiënten, en (2) het effect van behandeling met de steroid hormonen estradiol (E2) en progesteron (P) op de expressie van DNMTs en MBDs in explant kweken van proliferatief endometrium. De expressie van DNMTs en MBDs werd onderzocht met behulp van kwantitatieve real-time PCR (qRT-PCR).

De expressie van DNMT1 en MBD2 was significant hoger in secretoir endometrium (n=12) dan in proliferatief (n=8) en menstrueel endometrium (n=3). Daarnaast werd de expressie van DNMT1 en MBD2 significant ge-upreguleerd in endometrium dat behandeld was met een combinatie van E2+P, wat een rol voor DNMTs en MBDs bij de cyclische groei en differentiatie van het humane endometrium suggereert. Tenslotte was de expressie van verschillende DNMTs en MBDs significant lager in ectopische endometriose laesies dan in eutopisch endometrium van endometriose patiënten en controles zonder endometriose. Dit zou kunnen verklaren waarom veranderingen in promoter methylering van bepaalde genen gezien worden in ectopische endometriose laesies. Ook wordt het idee dat endometriose een epigenetische ziekte zou kunnen zijn door deze resultaten ondersteund.

In **Hoofdstuk 6** werd de hypothese getest dat de sterke up- of downregulatie van genen in eutopisch endometrium van endometriose patiënten in een eerder uitgevoerde microarray studie het gevolg is van methylering van de promoter. De door ons onderzochte genen werden geselecteerd op basis van biologische plausibiliteit en de aanwezigheid van een significant CpG eiland in de promoter van het gen. Zowel de expressie van mRNA als de methyleringsstatus van de onderzochte gebieden in het promoter CpG eiland van de genen PRIM2A, TGFβ3, EFN1, TAF6L, SHB, ITGA2, BENE, Rap1GAP, OGG1 en PRB was niet significant verschillend tussen eutopisch endometrium

van endometriose patiënten en eutopisch endometrium van controles. Vanwege recente nieuwe inzichten op het gebied van DNA methylering en het belang van zogenaamde “core regions” voor de regulatie van genexpressie is het op basis van deze resultaten helaas niet mogelijk om definitief een uitspraak te doen over de regulatie van deze genen door (promoter) methylering. Hiertoe bevelen we het opnieuw testen van onze hypothese aan met gebruikmaking van moderne, sensitieve, core region-specifieke technieken voor de detectie van DNA methylering aan in een grotere groep eutopische endometrium samples.

In **Hoofdstuk 7** worden de belangrijkste bevindingen samengevat en bediscussieerd in de context van recente literatuur. De beperkingen van onze studies en aanbevelingen voor toekomstig onderzoek worden vermeld.

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Curriculum Vitae

Kim van Kaam werd op 15 september 1977 geboren te Sittard. In 1995 behaalde ze cum laude haar gymnasiumdiploma aan het gymnasium van het toenmalige Bisschoppelijk College Sittard (thans: Trevianum scholengroep). In datzelfde jaar ving zij aan met de studie Geneeskunde aan de Universiteit Maastricht. Tijdens haar coschappen volgde zij een wetenschapsstage met als onderwerp de pathogenese van endometriose bij de vakgroepen Gynaecologie en Pathologie, met als begeleiders dr. A.F.P.M. de Goeij, dr. P.G. Groothuis en dr. G.A.J. Dunselman. Tevens volgde zij een keuze co-schap Obstetrie en Gynaecologie in het Onandjokwe Lutheran Hospital, Ondangwa, Namibië. Na het behalen van het artsexamen in 2001 werkte zij van december 2001 tot juni 2003 als arts-assistent niet in opleiding bij de afdeling Obstetrie en Gynaecologie in respectievelijk het Laurentius Ziekenhuis te Roermond en het Elkerliek Ziekenhuis te Helmond. Vervolgens werkte zij van juni 2003 tot oktober 2007 als IVF-arts/onderzoeker op de afdeling Obstetrie en Gynaecologie van het Maastricht Universitair Medisch Centrum. Tijdens deze periode werd aangevangen met het promotieonderzoek zoals beschreven in dit proefschrift. Het onderzoek werd uitgevoerd binnen het Maastrichtse onderzoeksinstituut GROW, School for Oncology and Developmental Biology, bij de afdeling Gynaecologie en Obstetrie. In oktober 2007 startte zij met de opleiding tot gynaecoloog. Deze werd aangevangen in het VieCuri Medisch Centrum te Venlo (opleider: dr. J.J. van Beek) en vanaf oktober 2009 voortgezet in het Academisch Ziekenhuis Maastricht (opleider: prof. dr. G.G.M. Essed, vanaf oktober 2010 prof. dr. R.F.P.M. Kruitwagen). In 2011 besloot zij haar carrière voort te zetten bij de afdeling klinische genetica. Per 1 september 2011 is zij gestart met de opleiding tot klinisch geneticus in het Maastricht Universitair Medisch Centrum (opleider: dr. C.E.M. de Die-Smulders).

Color figures

Figures chapter 2

Figure 1 Representative photographs of deep-invasive endometriotic lesions stained with antibodies against vimentin, alpha-smooth muscle actin (ASMA), desmin and smooth muscle myosin heavy chain (SM-myosin). Vimentin staining is strong in the stroma (Str) of the ectopic endometrium, whereas the cells surrounding the lesion (FM) are positive for ASMA. Some areas (M) are also positive for desmin and SM-myosin; most likely resident smooth muscle cells. Scale bar = 100 μ m.

Figure 3 Representative photographs of the negative controls for the vimentin, ASMA, desmin, SM-myosin, TGF- β receptor 1 and 2 and phosphorylated Smad immunostainings. Scale bar = 100 μ m.

Figure 4 Immunostaining of vimentin, ASMA, desmin and SM-myosin in submucosal connective tissue of the large bowel. Scale bar = 100 μ m.

Figure 5 Higher magnification photographs of an endometriotic lesion in the sigmoid, with fibromuscular tissue, stained for vimentin, ASMA, desmin and SM-myosin. Str = stroma in ectopic lesion, FM = fibromuscular tissue. Scale bar = 100 μ m.

Figure 6 Difference in fibromuscular tissue and structures that are most likely resident smooth muscle cells. The sections were stained for ASMA, desmin and SM-myosin. Str = stroma in ectopic lesion, FM = fibromuscular tissue, M = visceral muscle. Scale bar = 100 μ m.

Figure 7 A lesion in the visceral muscle of the colon wall. The stroma is positive for vimentin and ASMA, whereas the muscle cells are positive for ASMA, desmin and SM-myosin. Scale bar = 100 μ m

Figure 9 Representative photographs of immunostaining for TGF- β receptor 1 and 2 and phosphorylated Smad in deeply infiltrating endometriosis. Str = stroma in ectopic lesion, FM = fibromuscular tissue, GI = gland in ectopic lesion. Scale bar = 100 μ m

Figure 10 Vimentin and ASMA expression in endometriotic lesions generated through the subcutaneous transplantation of human endometrium in nude mice. Lesions were collected weekly for four weeks. The vimentin antibody is human-specific, which demonstrates that the ASMA expression is strongly enhanced in the host cells. Negative controls for the vimentin and ASMA stainings are shown in the inserts. Scale bar = 100 μ m

Figures chapter 4

Figure 3A Representative examples of heterogeneous expression of PR-A/B and PR-B protein in endometriotic lesions in non-carriers (patients carrying neither the +331A nor the PROGINS polymorphic allele)

Figure 3B Representative examples of heterogeneous expression of PR-A/B and PR-B protein in endometriotic lesions in patients carrying the PROGINS polymorphic allele. The left panel shows weak expression of PR-A/B and PR-B, whereas the right panel shows strong expression.

+331GA

PR-AB

PR-B

Figure 3C Representative examples of strong staining for PR-A/B and PR-B in endometriotic lesions of patients carrying the +331A polymorphic allele.

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