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A mi madre alegre, inteligente, hermosa y valiente

A mi padre idealista, luchador incansable y ejemplo académico

A mi abuela Gina, fuente de amor y alegría

A mi abuelo José Domingo Ramos Albornoz, el coronel honrado,
ético y valeroso, nuestro pilar, mejor amigo y abuelo de todos

A mi hermana Daniela, presente y futuro esplendor

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Abbreviations

AR	Androgen receptor
CI	Calcium ionophore
COC	Combined oral contraceptives
COX-2	Cyclooxygenase-2
E	Estradiol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
GnRH	Gonadotropin-releasing hormone
GnRH-a	Gonadotropin-releasing hormone agonists
HGF	Hepatocyte growth factor
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
IL-1RI	IL-1 receptor type 1
iNOS	Inducible nitric oxide synthase
IVF	<i>In vitro</i> fertilization
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide

MIF	Macrophage migration inhibitory factor
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
Mn-SOD	Manganese superoxide dismutase
NF-κB	Nuclear factor-kappa B
NIK	NF-κB inducing kinase
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical densitometry
P	Progesterone
PCB	Poly-chlorinated biphenyl
PPARγ	Peroxisome proliferator-activated receptor γ
PR	Progesterone receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
ROS	Reactive oxygen species
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor-α
TPA	Phorbol, 12-myristate, 13 acetate
TUNEL	TdT-mediated biotin-dUTP nick-end labeling
uPA	Urokinase type of plasminogen activator
VEGF	Vascular endothelial growth factor

Overview

Endometriosis is one of the most frequently encountered benign diseases in gynecology, being the cause of dysmenorrhea, dyspareunia, chronic pelvic pain and infertility in more than 35% of women of reproductive age. Decreased quality of life may result not only from the symptoms of pelvic pain and infertility, but also from the side effects of various medical and surgical treatments. For women with pain, surgery commonly provides temporary relief, but symptoms recur in up to 75% of women within 2 years, and further surgery is needed in many cases. The most widely accepted hypothesis on the origin of endometriosis is Sampson's theory of retrograde menstruation. According to this theory, endometriosis originates from endometrial cells regurgitated through the Fallopian tubes, which have the ability to survive, adhere, invade tissues, create a blood supply and proliferate outside their eutopic location. Endometriosis is nowadays considered to be a multifactorial and enigmatic disease. Anatomic, genetic, environmental, hormonal, immunologic and oxidative stress factors have been implicated in the establishment, development, maintenance and progression of endometriotic lesions

Local immunity has been found to be altered in peritoneal endometriosis. Abnormal levels of multiple cytokines and chemokines are present in the peritoneal fluid of endometriosis patients, resulting in a proinflammatory local environment. Cytokines, chemokines,

adhesion molecules and/or growth-promoting factors involved in endometriosis-associated inflammatory reaction include interleukin (IL)-1, IL-8, tumor necrosis factor (TNF)- α , regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, matrix metalloproteinase (MMP)-1 and macrophage migration inhibitory factor (MIF).

The transcription factor nuclear factor-kappaB (NF- κ B) plays a key role in the immune and inflammatory response, modulates cell proliferation and apoptosis in many cell types and has also been implicated in adhesion, invasion and angiogenesis. Interest in this molecule in the context of endometriosis has been steadily growing in recent years. To date, there is no direct evidence involving NF- κ B activity in the inflammatory response of endometriosis *in vivo*. Furthermore, the effect of NF- κ B activation or inactivation on cell proliferation and apoptosis in endometriosis, have not been previously studied. The aim of this study was to investigate the involvement and role of NF- κ B in peritoneal endometriosis *in vivo* and its potential as a novel central therapeutic target for the prevention and treatment of endometriosis.

Chapter I: Introduction

1. Endometriosis

1.1. Definitions

1.1.1. Endometrium and the menstrual cycle

Endometrial physiology

The endometrium is the internal layer of the uterus, composed of epithelial glands and stroma. The endometrium proliferates under the influence of estradiol (E) during the follicular phase (proliferative phase) of the ovulatory cycle (menstrual cycle). During the luteal phase (secretory phase), the endometrium is prepared for implantation by the action of progesterone (P). In case of fertilization, the blastocyst may implant in the endometrium upon its arrival in the uterus. If pregnancy fails to occur, the corpus luteum regresses and levels of P and E fall suddenly (Figure 1). P withdrawal is the physiological signal for menstruation. Progesterone receptor (PR)-positive stromal cells in the superficial zones of the endometrium respond to this decrease and initiate the menstrual cascade. This cascade involves increased production of prostaglandins generating vasoconstriction and hypoxia, greater expression of vascular endothelial growth factor (VEGF) and its receptor, elevated levels of stromal matrix metalloproteinases (MMPs), leukocytic invasion, and perivascular expression of cytokines and MMPs. MMPs have been

identified as the key class of proteinases involved in the initiation of menstruation (Marbaix *et al*, 1996; Brenner *et al*, 2002). During menstruation, the superficial endometrial zones are sloughed off. Menstrual endometrium is eliminated through the uterine cervix and vagina, but retrograde flow of the menstrual slough through the Fallopian tubes occurs in most women (Halme *et al*, 1984).

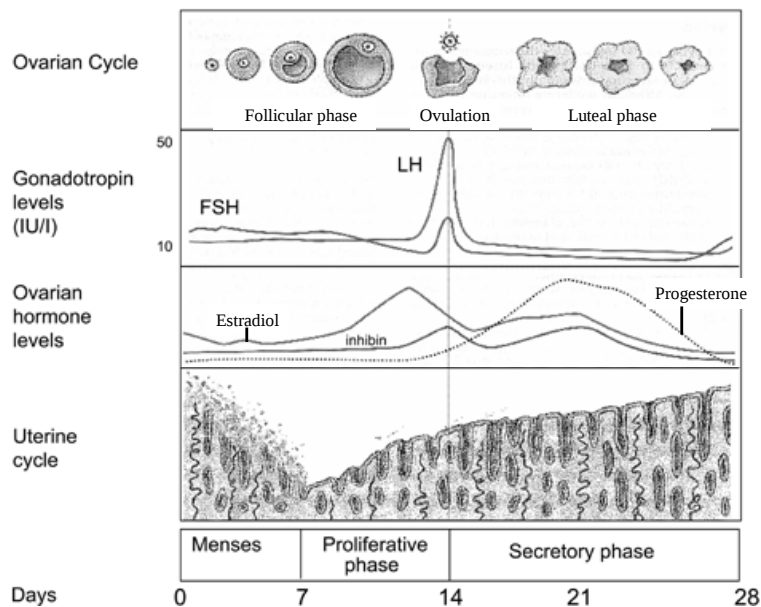


Figure 1: Menstrual cycle. FSH: follicle-stimulating hormone, LH: luteinizing hormone (adapted from Kaplan and Manuck, 2004).

Endometrial histology

Human endometrium has two major components: endometrial glands and stroma (Figure 2). Endometrial glands consist of simple columnar

epithelium (epithelial cells) forming numerous tubular glands, which are supported by endometrial stroma. Endometrial stroma is composed of a variety of cells (stromal cells), including spindle-shaped connective tissue cells and bone marrow-derived cells. Both glands and stroma undergo extensive changes during the menstrual cycle.

Two layers have been characterized in the endometrium:

- a. A superficial layer or stratum functionalis that is sloughed off during menstruation and redevelops with each new cycle.
- b. A deep layer or stratum basalis composed of permanent stromal tissue and the blind ends of the uterine glands. The stratum basalis is maintained permanently during the menstrual cycle and provides the stratum functionalis with a cell source.

Endometrial glands have quite a flat form and mitotic cells are often found during the proliferative phase. During the secretory phase, epithelial glands are composed of mature secretory cells and their shape is more tortuous (Figure 2).

During the proliferative phase, 10-15% of cells in the stroma are positive for the leukocyte-common antigen CD45, increasing to 20-25% in the late secretory phase. T cells and macrophages are the major leukocyte populations in the stroma, with T cells most often found in aggregates within the deeper layers of the endometrium, and macrophages scattered throughout the endometrium. The density of

macrophages increases during menstruation (Fernández-Shaw *et al*, 1995).

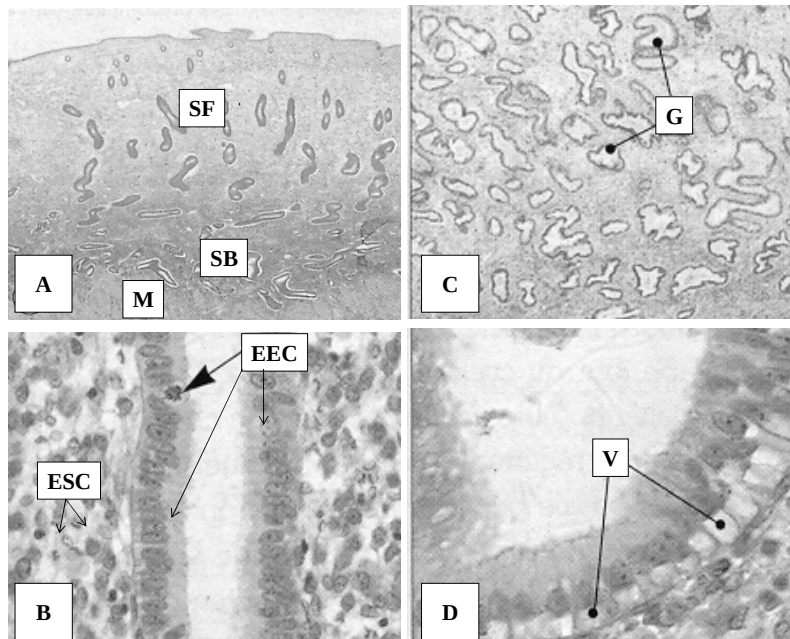


Figure 2: Histology of endometrium. Pictures A and B show endometrial histology during the proliferative phase of the menstrual cycle, and C and D during the secretory phase. SF: stratum functionalis, SB: stratum basalis, M: myometrium, G: endometrial glands, EEC: endometrial epithelial cells, ESC: endometrial stromal cells, V: vacuoles (adapted from Stevens and Lowe, 1997).

Another important component of the endometrial stroma is the vascular network, which also participates in the menstrual cycle. During menstruation, distal vessels are sloughed off, while spiral arteries retract into the stratum basalis and constrict to limit blood loss. During the proliferative phase, spiral arteries extend again as the stratum functionalis redevelops.

1.1.2. Endometriosis

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterine cavity. The most common locations are the pelvic peritoneum and the ovaries. Extraperitoneal endometriosis also exists, notably in the rectovaginal septum. Indeed, this disorder can be found in all organs, except the spleen (Markham *et al*, 1989; Nisolle and Donnez, 1997).

1.2. Epidemiology

Endometriosis is one of the most frequently encountered benign diseases in gynecology. The prevalence of endometriosis may vary depending on the study. In asymptomatic women undergoing tubal ligation, the prevalence is about 4% (1-7%). In women with primary infertility, the prevalence varies from 9 to 50%, while in women with pelvic pain, it ranges from about 5 to 21%. Around 50% of adolescents with intractable dysmenorrhea or pelvic pain are found to have endometriosis (Cramer and Missmer, 2002; Donnez *et al*, 2002a).

The most common risk factors for endometriosis are the presence of dysmenorrhea (which may be interpreted as a symptom of disease), early menarche (defined as $\leq$ age 11) and a shorter cycle length (defined as $\leq$ 27 days). Some studies suggest that a longer-lasting and heavier menstrual flow may favor the development of endometriosis, and outflow obstruction of menstruation might alone be sufficient to

cause endometriosis (Cramer and Missmer, 2002; Donnez *et al*, 2002a).

Other risk factors include taller height, alcohol and caffeine consumption, family history, and dioxin-like polychlorinated biphenyls (PCB) or dioxin exposure. Higher parity and weight, regular exercise and smoking may decrease the risk for developing endometriosis (Cramer and Missmer, 2002; Donnez *et al*, 2002a).

1.3. Clinical symptoms and diagnosis

Endometriosis is the cause of dysmenorrhea (pelvic pain during menstruation), dyspareunia (pain during sexual intercourse), chronic pelvic pain and infertility in more than 35% of women of reproductive age. Endometriotic lesions on the anterior cul-de-sac or bladder flap may give rise to dysuria, urinary urgency, pollakiuria, suprapubic pain and hematuria when there is invasion through the bladder wall. Involvement of the ureter may occur with or without obstruction, and may lead to the loss of a kidney.

The most common form of extraperitoneal endometriosis involves the rectovaginal septum, and may present as intestinal obstruction, rectal pain, distension, diarrhea, constipation and/or rectal bleeding. Lung and chest wall endometriosis usually present as pneumothorax, hemothorax or hemoptysis.

Endometriosis is most often diagnosed in women of reproductive age. Although rare, endometriosis may occur during the menopause. In

asymptomatic women, the diagnosis of endometriosis ranges from 2 to 22% of reproductive-age women (Donnez *et al*, 2002a; Murphy, 2002).

Time to diagnosis can be very long (mean 11.7 years in the USA and 8 years in the UK) because of the variability in symptoms and signs, and confusion with other disorders. The gold standard for diagnosis of pelvic disease is surgical assessment by laparoscopy or laparotomy and biopsy. A scoring system has been developed to assess the extent of disease: the revised American Society for Reproductive Medicine (rASRM) classification of endometriosis (Anonymous, 1997).

Decreased quality of life may result not only from the symptoms of pelvic pain and infertility, but also from the side effects of various medical and surgical treatments. For women with pain, surgery commonly provides temporary relief, but symptoms recur in up to 75% of women within 2 years, and further surgery is needed in many cases (Giudice and Kao, 2004).

1.4. Peritoneal, ovarian, and rectovaginal endometriosis are three distinct entities

The most widely accepted hypothesis on the origin of endometriosis is Sampson's theory of retrograde menstruation (Sampson, 1927). According to this theory, endometriosis originates from endometrial cells regurgitated through the Fallopian tubes (Figure 3).

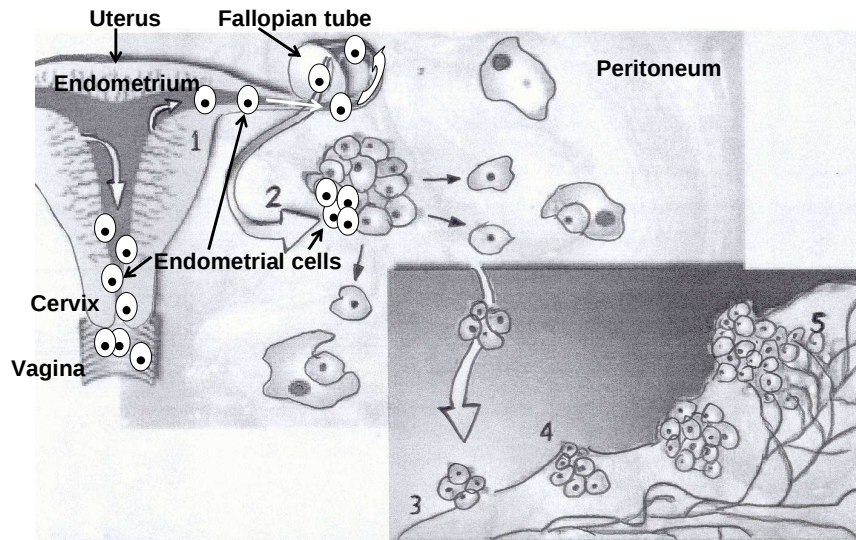


Figure 3: Sampson's theory. 1) During menstruation, the superficial layer of the endometrium is sloughed off through the uterine cervix and vagina, but retrograde flow of the menstrual slough through the Fallopian tubes may occur. 2) Regurgitated endometrial cells arrive in the peritoneal cavity. 3) Endometrial cells adhere to the peritoneum. 4) The peritoneum is invaded by endometrial cells. 5) Establishment of a blood supply, cell proliferation and resistance to apoptosis in endometrial cells all play a role in the development of peritoneal endometriotic lesions (adapted from Groothuis *et al*, 2005).

In 1996, Donnez *et al* distinguished two different types of endometriosis. The rectovaginal endometriotic nodule was then classed as a distinct entity from peritoneal endometriosis. One year later, Nisolle and Donnez (1997) published a definitive guide for future publications on endometriosis: "Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities". In this paper, peritoneal

endometriosis was explained by the retrograde menstruation theory, and peritoneal endometriotic lesions were characterized depending on their appearance into red, black or white endometriotic lesions (Figure 4).

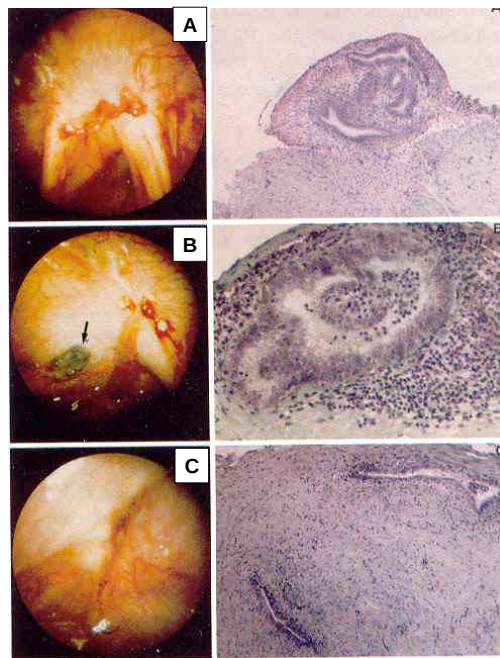


Figure 4: Peritoneal endometriosis. (A) Red endometriotic lesion at laparoscopy. Numerous glands with active epithelium and abundant stroma are found on the peritoneal surface. (B) Typical black endometriotic lesion: combination of glands, stroma and intraluminal debris. (C) White endometriotic lesion: occasional retroperitoneal glandular structures and scanty stroma (Nisolle and Donnez, 1997).

Morphologic and morphometric data showed similarities between eutopic endometrium and red peritoneal lesions, and the evolution theory, demonstrating the transition from red to black and then to white lesions, was proposed (Figure 5).

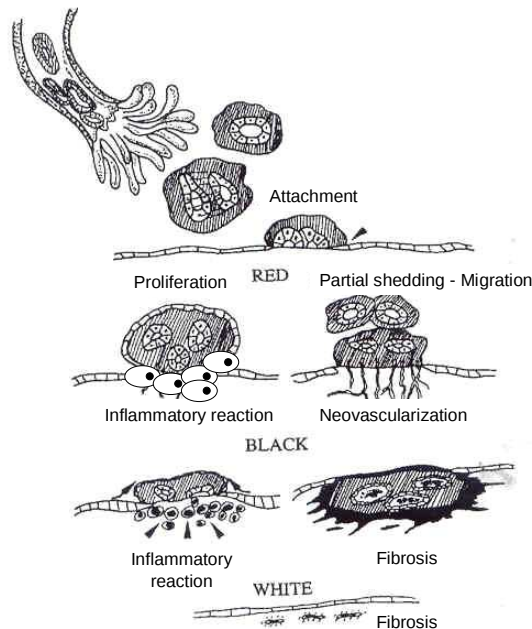


Figure 5: Evolution theory of peritoneal endometriosis (adapted from Nisolle and Donnez, 1997).

Concerning the pathogenesis of ovarian endometriosis, the presence of mesothelial invagination in continuum with endometriotic tissue suggested that metaplastic histogenesis of ovarian endometriotic lesions occurs, but endometriotic cysts may also originate from invagination of superficial endometriotic implants. Regarding the origin of rectovaginal nodules, immunocytochemical results showed poor differentiation and hormonal independence of these lesions and indicated a close relationship with their mesodermal Müllerian origin. Histologically, scanty endometrial-type stroma and glandular epithelium were found to be disseminated in muscular tissue, showing

striking similarities to adenomyomas or adenomyotic nodules, originating in the retroperitoneal space (Donnez and Squifflet, 2004). Others suggested that rectovaginal nodules could originate from deep-infiltrating endometriosis from the pelvic peritoneal cavity (Koninckx and Martin, 1992). Hematological spread of endometrial cells and metaplasia of different cell types were also proposed as theories to explain extrapelvic endometriosis in the lungs or other organs.

The importance of discriminating between these three different entities was duly confirmed during subsequent years of research into endometriosis, since most studies on endometriosis following this publication focused their objectives distinguishing between peritoneal endometriosis, ovarian endometriosis and adenomyotic nodules of the rectovaginal septum.

For the purposes of this study, research was conducted into peritoneal endometriosis.

1.5. Origin and establishment of peritoneal endometriotic lesions

Sampson's theory (Figure 3) explains the origin of endometriosis as the consequence of reflux of endometrial fragments through the Fallopian tubes during menstruation, with subsequent implantation and growth on the peritoneum (Sampson, 1927). This is the most widely accepted theory to explain the occurrence of peritoneal endometriosis.

Sampson's transplantation theory is supported by the high prevalence of pelvic endometriosis in girls with congenital obstruction of their menstrual outflow. Menstruation is often longer and heavier in women with endometriosis (Sanfilippo *et al*, 1986; Darrow *et al*, 1993; Vercellini *et al*, 1997; Vinatier *et al*, 2001) and cycles tend to be shorter (Arumugam and Lim, 1997). Furthermore, obstruction of antegrade menstruation and the formation of uteropelvic fistulas are effective ways to induce endometriosis in primate models, clearly indicating that increased retrograde menstrual flow increases the risk of developing endometriosis (Te Linde and Scott, 1950; D'Hooghe *et al*, 1994).

Retrograde menstruation may occur in up to 90% of patients with patent tubes (Halme *et al*, 1984), but not all of them will develop endometriosis. Peritoneal reflux appears to be necessary, but not sufficient in itself, for the development of peritoneal endometriosis. In the majority of women, the peritoneal environment is able to eliminate refluxed endometrial tissue, but this cleansing mechanism seems to be deficient in women who develop endometriosis. Thus, the origin and establishment of endometriosis is explained by the ability of endometrial cells to survive, adhere, invade tissues, create a blood supply and proliferate outside their eutopic location (Van Langendonckt *et al*, 2002a; Giudice and Kao, 2004).

1.5.1. Adhesion to the peritoneal surface

Menstrual endometrium with viable endometrial cells may therefore enter the pelvic cavity and come into close contact with the peritoneum, creating constant apposition of endometrium to mesothelium. *In vitro*, the mesothelial lining can be damaged by the contents of the menstruum, and the extracellular matrix (ECM) is thus exposed, forming adhesion sites for endometrial tissue (Dunselman *et al*, 2001). *In vivo*, endometrial cells have been shown to adhere to intact mesothelium in a murine model (Nisolle *et al*, 2000a, 2000b; Donnez and Nisolle, 2001). Adhesion of endometrial cells to the peritoneum observed *in vivo* could be explained by damage or remodeling of the mesothelium induced by the menstrual tissue, as demonstrated in *in vitro* experiments (Koks *et al*, 2000; Demir Weusten *et al*, 2000).

Integrins and cadherins are two important mediators of cell-cell and cell-matrix adhesion and these cell adhesion molecules are found on the endometrium, in menstrual effluent and in endometriotic tissue samples (Van der Linden *et al*, 1994, 1995; Bridges *et al*, 1994; Kim and Yamada, 1997; Regidor *et al*, 1998).

1.5.2. Invasion of the epithelium

The initial establishment of endometriosis is an invasive event requiring ECM breakdown. Endometrial stromal and epithelial cells have been implicated in the attachment and early invasion of the peritoneum. Two families of proteolytic enzymes are involved in this

process: MMPs and the plasminogen/plasmin activation system (Rodgers *et al*, 1994). Once endometrial tissue adheres to the ECM, endometrial metalloproteinases begin active remodeling of the ECM, leading to endometrial invasion of the submesothelial space of the peritoneum (Nisolle *et al*, 2000a; Dunselman *et al*, 2001; Witz *et al*, 2003). MMPs have been shown to play a key role in the initiation of menstruation and contribute to the implantation and further invasion of seeded endometriotic explants (Marbaix *et al*, 1996; Singer *et al*, 1997; Henriët *et al*, 2002). A direct correlation between MMP-1 expression and the activity of endometriotic foci has been reported (Kokorine *et al*, 1997). Suppressing MMP secretion with progesterone treatment or blocking enzyme activity with the tissue inhibitor of metalloproteinase-1 (TIMP-1) was able to prevent the formation of ectopic lesions in a nude mouse model of endometriosis (Bruner *et al*, 1997).

The second family of proteases involved in peritoneal invasion of endometrial tissue is the plasminogen/plasmin system. Plasminogen and urokinase-type plasminogen activator (uPA) have been detected at higher concentrations in ectopic than eutopic endometrium (Sillem *et al*, 1998). Another molecule that has been shown to enhance stromal cell invasion is hepatocyte growth factor (HGF). HGF stimulates stromal cell invasion partly through uPA induction (Yoshida *et al*, 2004).

1.5.3. Angiogenesis

The development of a blood supply through angiogenesis has been proposed as an essential process for the establishment, survival and growth of endometriotic lesions. Angiogenesis involves proliferation, migration and extension of endothelial cells, adherence of these cells to the ECM, remodeling of the ECM, and formation of a new lumen (Folkman and Shing, 1992; Donnez *et al*, 1998). Menstrual effluent contains high concentrations of vascular endothelial growth factor (VEGF), one of the main factors stimulating angiogenesis. Increased levels of angiogenic factors and angiogenic activity have been detected in the peritoneal fluid of women with endometriosis (Oosterlynk *et al*, 1993; Koninckx *et al*, 1998; McLaren, 2000). The presence of VEGF has been confirmed in epithelial glands, stromal cells and macrophages from ectopic endometrium. The high VEGF levels found in endometriotic lesions could produce an increase in the subperitoneal vascular network and facilitate the initial development and maintenance of endometriotic lesions (McLaren *et al*, 1996; Shifren *et al*, 1996; Donnez *et al*, 1998).

1.5.4. Endometriotic lesion survival

Cell proliferation is a fundamental process in the development of endometriotic lesions. In the nude mouse model of endometriosis, Nisolle *et al* (2000a) demonstrated that shortly after attachment of stromal cells, a rearrangement of epithelial and stromal cells occurs, leading to the development of endometriotic lesions and cystic glands

within just 5 days. Extensive proliferation was observed in glandular cells as early as 3 days after transplantation. The growth of endometriotic tissue can be regulated by ovarian steroid hormones and a number of cytokines and growth factors such as interleukin (IL)-6, IL-8, tumor necrosis factor α (TNF- α), and HGF (Harada *et al*, 2001; Khan *et al*, 2003).

HGF, its receptor c-Met, the proliferating cell nuclear antigen, and survivin mRNA have all been found to be expressed in peritoneal endometriotic lesions (Khan *et al*, 2003; Fujino *et al*, 2006), conferring an antiapoptotic and mitogenic phenotype to these lesions. Ectopic and regurgitated endometrial cells show resistance to apoptosis, contributing to the development of endometriotic lesions (García-Velasco and Arici, 2003; Beliard *et al*, 2004).

1.5.5. Evolution, activity and appearance of peritoneal endometriotic lesions

Peritoneal lesions go through various stages and have a range of aspects, appearing as red, black or white lesions (Figures 4 and 5), red lesions being the most active in terms of cell proliferation, inflammatory response and vascularization (Nisolle and Donnez, 1997; Donnez *et al*, 1998; Khan *et al*, 2004; Van Langendonck *et al*, 2004). The morphology of red peritoneal lesions is similar to that of eutopic endometrium, suggesting that these lesions are the first stage of early implantation of endometrial glands and stroma (Figure 5). After partial shedding, red lesions can regrow constantly. This

shedding induces an inflammatory reaction, provoking scarification, and the lesions become black. The subsequent fibrosis leads to areas of white opacification that are inactive (Nisolle and Donnez, 1997).

Other studies have shown differential inflammatory reactions, vascularization, and mitotic and apoptotic activity in red and black endometriotic lesions. The more intense inflammatory response observed in red endometriotic implants is substantiated by findings that macrophage infiltration, MMP-1 mRNA expression and macrophage migration inhibitory factor (MIF) are increased in red endometriotic lesions compared to black lesions (Kokorine *et al*, 1997; Kats *et al*, 2002; Khan *et al*, 2004). A recent study by Lawson *et al* (2007) showed higher expression of IL-1 receptor type 1 (IL-1RI) in red endometriotic lesions than in black and in white lesions. IL-1RI could be one of the factors responsible for the increased inflammatory response in red endometriotic lesions.

The observation of a more extensive vascularization pattern in red endometriotic lesions is supported by the fact that VEGF and microvessel density have been found to be higher in red endometriotic lesions than black lesions (Donnez *et al*, 1998; Khan *et al*, 2003).

Concerning the mitotic and apoptotic activities of peritoneal endometriotic lesions, hepatocyte growth factor (HGF), its receptor c-Met, the proliferating cell nuclear antigen, and survivin mRNA expression and concentrations have also been found to be greater in

red endometriotic lesions than black lesions (Khan *et al*, 2003; Fujino *et al*, 2006).

All these findings confer to red endometriotic lesions a major status of inflammation, vascularization, proliferation and resistance to apoptosis.

1.6. Endometriosis: a multifactorial disease

Endometriosis is nowadays considered to be a multifactorial disease. In addition to the etiopathogenic factors previously mentioned, genetic, environmental, hormonal, immunologic and oxidative stress factors have also been implicated in the establishment, development, maintenance and progression of endometriotic lesions (Figure 6).

1.6.1. Genetic factors

Endometriosis is a condition showing hereditary tendencies and a polygenic/multifactorial etiology has been suggested. A number of candidate genes have been proposed with potential biological plausibility (cytochrome P450, N-acetyl transferase 2, glutathione-S-transferase, galactose-1-phosphate uridyl transferase, ER, PR, AR, p53, PTEN, PPAR γ 2). Some of these genes point to abnormalities in detoxification enzymes, which could lead to vulnerability to environmental stimuli, while others (tumor suppressor genes) are associated with malignant transformation (Simpson and Bischoff, 2002; Giudice and Kao, 2004). New findings reported by Zondervan *et al* (2007) provide significant evidence that a locus with near-

Mendelian autosomal inheritance on chromosome 7p is linked to endometriosis.

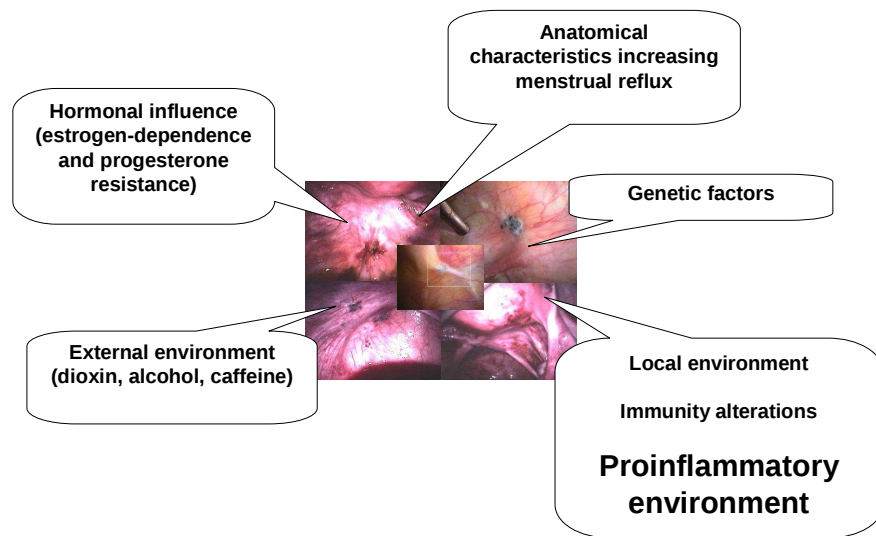


Figure 6: Endometriosis: a multifactorial disease.

1.6.2. Environmental exposure

Endocrine-disrupting compounds, such as dioxins or dioxin-like PCBs, could play a role in the establishment or development of endometriosis. Experimental data in monkeys exposed to dioxin have shown development of endometriosis in a dose-dependent manner. Belgium, with the highest levels of dioxin pollution in the world, has the highest incidence of endometriosis, as well as the highest prevalence of severe endometriosis (Rier *et al*, 1993; Koninckx *et al*,

1994; Heilier *et al*, 2005). Heilier *et al* (2005) provided the first epidemiological evidence of a clear association between increased dioxin and dioxin-like PCB impregnation and the risk of endometriosis.

1.6.3. Hormonal factors

Endometriosis is known to be an estrogen-dependent disease, but progesterone (P) resistance has also been postulated to be a mechanism of progression of endometriosis (Bulun *et al*, 2006).

Endometriotic lesions show high estradiol (E) biosynthesis and low E inactivation compared to endometrium from unaffected women. The central enzyme in the biosynthesis of estradiol is aromatase, which catalyzes the conversion of androgens to estrogens. Aromatase is normally absent from endometrium, but abnormal expression of aromatase has been demonstrated in endometriotic lesions and, at much lower levels, the endometrium of women with endometriosis. In addition, inactivation of E by 17 β -hydroxysteroid dehydrogenase 2 is impaired due to a lack of this enzyme in glandular cells in endometriosis, leading to increased local concentrations of E (Noble *et al*, 1997; Zeitoun *et al*, 1998, 1999; Zeitoun and Bulun, 1999)

Expression of both PR-A and PR-B isoforms appears to be dysregulated in the endometrium of endometriosis patients, while in endometriotic implants, only the A isoform is expressed. The presence of PR-A (inhibitory isoform) and absence of PR-B (stimulatory

isoform) has been proposed to explain P resistance in endometriotic lesions. Since P inhibits and E stimulates the growth of endometriotic lesions, partial progesterone resistance and increased local concentrations of E have been suggested as important elements in the development of endometriosis (Lessey *et al*, 1989; Nisolle *et al*, 1997; Berqvist and Ferno, 1999; Attia *et al*, 2000).

1.6.4. Immunologic factors

Local immunity has been found to be altered in peritoneal endometriosis. Abnormal levels of multiple cytokines and chemokines are present in the peritoneal fluid of endometriosis patients, resulting in a proinflammatory local environment. Activated macrophages showing reduced surface expression of scavenger receptors have been reported to be increased in the peritoneal cavity of women with endometriosis, leading to increased cytokine production, but decreased phagocytic activity. Increased cytokine levels and altered macrophage function can promote the survival and growth of ectopic endometrial cells (Harada *et al*, 2001; Sidell *et al*, 2002; Van Langendonck *et al*, 2002a).

Cytokines, chemokines, adhesion molecules and/or growth-promoting factors involved in endometriosis-associated inflammatory reaction include IL-1, IL-8, TNF- α , IFN- γ (interferon- γ), RANTES (regulated on activation, normal T cell expressed and secreted), MCP-1 (monocyte chemoattractant protein-1), ICAM-1, HGF, MMP-1 and MIF. In the peritoneal fluid of women with endometriosis, these

molecules can originate from endometriotic implants, macrophages or other immune cells (Harada *et al*, 2001; Chegini, 2002; Gazvani and Templeton, 2002; Giudice and Kao, 2004).

Natural killer cell activity is also altered in the peritoneal fluid of women with endometriosis, leading to decreased surveillance of ectopic tissue (Oosterlynck *et al*, 1991).

1.6.5. Oxidative stress

Several studies suggest that oxidative stress is a constituent of the endometriosis-associated inflammatory response. Retrograde menstruation carries erythrocytes, apoptotic endometrial tissue and cell debris into the peritoneal cavity, components which, in conjunction with peritoneal macrophages, contain highly pro-oxidant factors such as heme and iron, well known inducers of oxidative stress. Reactive oxygen species (ROS) may play a role in regulating expression of genes encoding immunoregulators, cytokines and cell adhesion molecules implicated in the pathogenesis of endometriosis (Arumugam and Yip, 1995; Murphy *et al*, 1998).

Previous studies conducted by our team at the Université Catholique de Louvain have shown increased iron levels in peritoneal tissue, macrophages, pelvic fluid and ectopic endometrial tissue from patients with endometriosis (Van Langendonck *et al*, 2002a, 2002b, 2002c, 2004). Increased levels of iron are associated with tissue damage. Iron can act as a catalyst to potentiate oxygen toxicity by generating a wide

range of free radical species. A recent publication by our group and PhD thesis entitled “Study of the potential involvement of iron in the pathogenesis of peritoneal endometriosis” by S. Defrère (2006) established that iron overload contributes to the further growth of endometriosis by promoting epithelial cell proliferation in endometriotic lesions (Defrère *et al*, 2006).

1.7. Current therapies and novel therapeutic strategies in endometriosis

Current therapies

Endometriosis therapy has essentially three main objectives: to reduce pain, to increase the possibility of pregnancy and to delay recurrence for as long as possible.

A number of drugs have been shown to diminish endometriosis-associated pain: non-steroidal anti-inflammatory drugs (NSAIDs), combined oral contraceptives (COCs), danazol, gestrinone, medroxyprogesterone acetate and gonadotropin-releasing hormone agonists (GnRH-a). They show similar efficacy, but their side effects and cost profiles differ (Donnez *et al*, 2002a, 2002b, 2004).

There is general agreement that visible endometriosis should be removed at the time of surgery. Danazol or GnRH-a therapy for 6 months after surgery reduces endometriosis-associated pain and delays recurrence rates at 12 and 24 months (Donnez *et al*, 2002a; Kennedy *et al*, 2005).

In moderate and severe endometriosis-associated infertility, the combined approach (operative laparoscopy with GnRH-a for 3 months) is considered as first-line treatment. After surgery, the mean pregnancy rate is about 50% (Donnez *et al*, 2002a, 2002b). *In vitro* fertilization (IVF) is indicated as second-line treatment in patients who fail to become pregnant naturally after surgical treatment or as first-line treatment if tubal function is compromised or male factor infertility is established (Donnez *et al*, 2002a; Kennedy *et al*, 2005).

The rectovaginal adenomyotic nodule, considered as a distinct entity, requires a surgical approach because of the poor response to any medical therapy (Koninckx and Martin, 1992; Donnez *et al*, 2002a, 2002b; Donnez and Squifflet, 2004).

Novel therapeutic strategies

Novel therapeutic strategies are based on many of the molecular targets mentioned earlier, including hormonal therapies, inflammatory and immunological factors, antioxidants, metalloproteinases and angiogenesis. Emerging hormonal therapies include aromatase inhibitors, GnRH antagonists, progestogen agonists, selective PR modulators, selective ER modulators and ER- β agonists. Novel non-hormonal therapies include antiangiogenic agents and anti-inflammatory agents such as selective cyclooxygenase-2 (COX-2) inhibitors, TNF- α inhibitors and PPAR- γ agonists (Giudice and Kao, 2004; D'Hooghe *et al*, 2006; Mihalyi *et al*, 2006).

The absence of any wholly successful medical or surgical therapy, the unacceptable side effects provoked by long-term medical treatments, the epidemiological and social impact of endometriosis and the disability suffered by each individual patient make continued research in this field absolutely indispensable to fully understand the mechanisms implicated in endometriosis development, and to discover new central targets for the treatment of this debilitating disease.

In the next section, the transcription factor nuclear factor-kappaB (NF- κ B) will be introduced. This factor plays a key role in the immune and inflammatory response, modulates cell proliferation and apoptosis in many cell types and has also been implicated in angiogenesis. Interest in this molecule in the context of endometriosis has been steadily growing in recent years.

The involvement of NF- κ B in peritoneal endometriosis and its potential as a novel central therapeutic target for the treatment of endometriosis will be investigated.

2. The transcription factor nuclear factor-kappaB (NF-κB)

2.1. The NF-κB-signaling system

More than 24000 articles appear in PubMed when the word NF-kappaB is keyed in, giving us some idea of the magnitude and importance of this transcription factor in scientific research, since its discovery by Sen and Baltimore (1986) over 20 years ago.

NF-κB is a transcription factor that plays a crucial role in inflammation, immunity, cell proliferation and apoptosis. It is involved in several pathologies and is known to play a key role in the transduction of proinflammatory signals. The NF-κB pathway has been shown to be modulated in a cell-specific manner and to interact with other transcription pathways, providing a complex variety of cellular responses to its activation (Viatour *et al*, 2005; Perkins, 2007).

NF-κB activation

NF-κB peptides are assembled through the dimerization of five subunits: p50/p105 (NF-κB1), p52/p100 (NF-κB2), p65 (RelA), c-Rel and RelB (Figure 7), the most extensively studied dimer being p50/p65 (Karin, 2006).

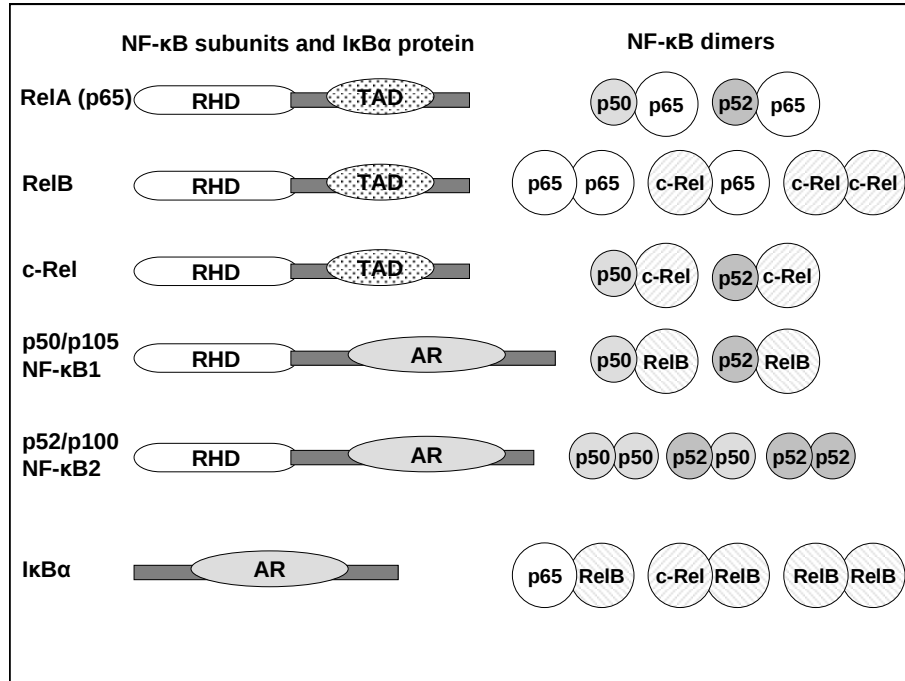


Figure 7: NF-κB subunits and dimers and IκBα protein. RHD: Rel-homology domain, responsible for DNA binding, dimerization and association with IκB proteins. TAD: transcriptional activation domain. AR: ankyrin repeats, responsible for cytoplasmic retention and inhibition of NF-κB. The peptides p50 and p52 derive from the precursor proteins p105 and p100 and lack a TAD; therefore dimers in the 5th row lack TADs. Dimers in the final row are not able to bind to DNA (adapted from Karin *et al*, 2004 and Hoffmann and Baltimore, 2006).

NF-κB dimers are located mostly in the cytoplasm in an inactive form, binding to specific NF-κB inhibitors, IκB proteins, which prevent NF-κB-DNA binding (Figure 8). Diverse stimuli may activate the IκB kinase (IKK) complex, which phosphorylates NF-κB-coupled IκB peptides, inducing their polyubiquitination and rapid degradation by the 26S proteasome. Thus, liberated NF-κB dimers, capable of binding to DNA, translocate to the nucleus, activating the transcription of

several target genes (Baldwin, 1996; Barnes, 1997; DiDonato *et al*, 1997; Lawrence *et al*, 2001; Karin *et al*, 2004; Hoffmann and Baltimore, 2006). NF- κ B activity is constitutive in B cells and in some monocyte cell lines but, in most other normal cells, it is very low or undetectable (Rice and Ernst, 1993). Most tumor cell lines and tumor tissues derived from patients with different types of cancer also show constitutive activation of NF- κ B (Aggarwal, 2004).

NF- κ B activation pathways

Different NF- κ B activation pathways have been described (Figure 8). The classic or canonical pathway is induced by proinflammatory stimuli such as pathogen-derived lipopolysaccharide (LPS) and cytokines such as TNF- α and IL-1. This pathway is dependent on IKK β activation, which phosphorylates I κ B α and mainly results in the activation of p50/p65 heterodimers.

The alternative or non-canonical pathway is induced by several stimuli (CD40 and lymphotoxin- β receptors, B-cell-activating factor of the TNF family, LPS and latent membrane protein-1) and is dependent on IKK α activation, resulting in the activation of p52/RelB heterodimers.

The canonical pathway is principally responsible for regulating inflammation and control of proliferation and apoptosis of lymphoid cells during the immune response, being fast-acting (responds within minutes) and reversible, due to the presence of multiple negative

feedback mechanisms. The non-canonical pathway is related to lymphoid organogenesis and responds slowly (over hours and days), ensuring long-lasting NF- κ B activity.

Atypical pathways may be IKK-dependent or -independent, activating casein kinase-II or tyrosine kinase depending on the stimuli, and may induce differentially modified forms of NF- κ B subunits with distinct functions (Hoffmann and Baltimore, 2006; Perkins, 2007).

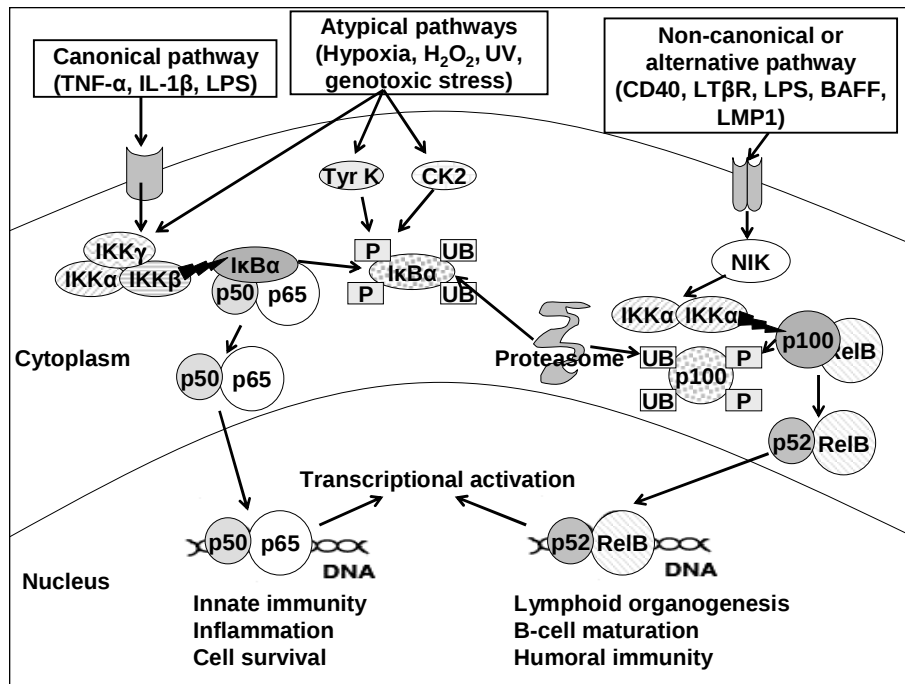


Figure 8: NF- κ B activation pathways. UV: ultraviolet radiation, LT β R: lymphotoxin- β receptor, BAFF: B-cell-activating factor of the TNF family, LMP1: latent membrane protein-1, Tyr K: tyrosine kinase, CK2: casein kinase-II, P: phosphorylation, UB: ubiquitination, NIK: NF- κ B-inducing kinase (adapted from Bonizzi and Karin, 2004, and Perkins, 2007).

NF- κ B-regulated genes

NF- κ B transcriptional activity regulates many genes implicated in inflammation (IL-1, IL-6, IL-8, iNOS, COX-2), immune regulation (IFN- γ , TNF- α , RANTES, ICAM-1), apoptosis and cell proliferation (Bax, Fas, Fas-L, cyclin D1, c-Myc, EGF) and angiogenesis (VEGF) (Figure 9).

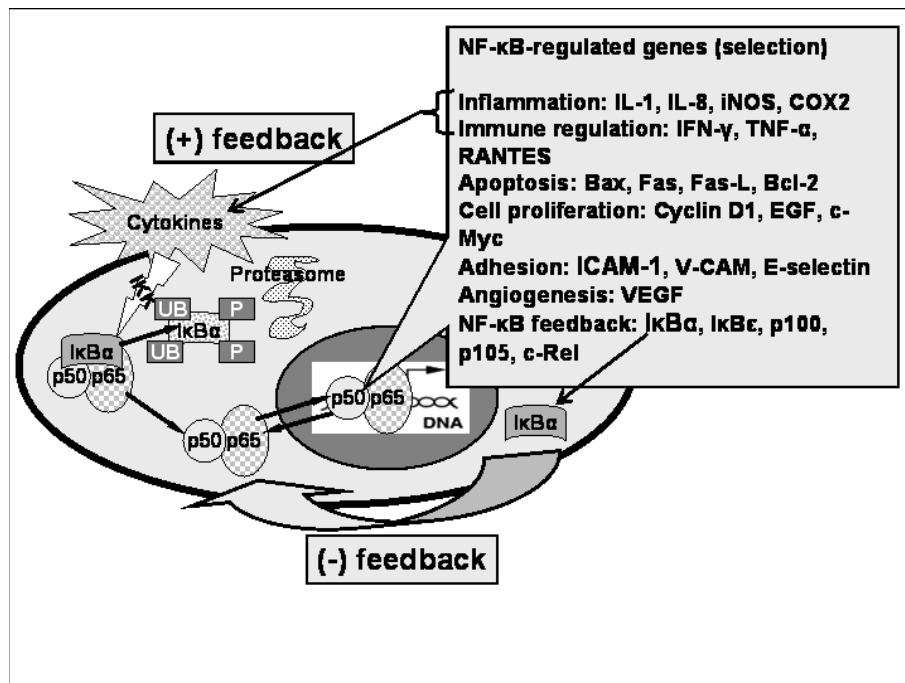


Figure 9: NF- κ B-regulated genes with positive and negative feedback modulating the NF- κ B pathway.

NF- κ B regulation involves negative feedback inhibition through NF- κ B-directed synthesis of I κ B α , p100 and/or p105 (Chiao *et al*, 1994; Aggarwal, 2004; Karin, 2006) (Figure 9). I κ B α enters the nucleus,

removes NF- κ B from the DNA, and then exports it to the cytoplasm, restoring the pool of inactive NF- κ B/I κ B α complexes (Arenzana-Seisdedos *et al*, 1997). In pre-B or mature B cells, there is a balanced state between the rates of degradation and synthesis of I κ B α proteins (Rice and Ernst, 1993).

2.2. NF- κ B, immunity and inflammation

Multiple functions have been ascribed to NF- κ B in the immune system, including regulation of the development, differentiation and survival of immune cells, as well as regulation of local and systemic immune activities (Hoffmann and Baltimore, 2006).

NF- κ B activation has been implicated in many inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, lupus, inflammatory bowel disease, sepsis, and others (Neurath *et al*, 1998; Abraham, 2003; Roman-Blas and Jimenez, 2006).

A central role of NF- κ B in the immune system involves modulation of inflammation. As previously mentioned, NF- κ B is activated by proinflammatory stimuli, such as TNF, IL-1, IL-12, MCP-1 and RANTES, inducing expression of multiple genes encoding proinflammatory and/or immunomodulatory cytokines, chemokines, growth factors and adhesion molecules like IL-1, IL-6, IL-8, iNOS, COX-2, IFN- γ , TNF- α , RANTES and ICAM-1. Almost all genes induced by proinflammatory stimuli are upregulated by NF- κ B (www.nf-kb.org; Hoffmann and Baltimore, 2006).

Both initiation and resolution of inflammation have been linked to NF- κ B transcriptional activity. This could be related to the negative feedback mechanisms regulating the activity of NF- κ B. In macrophages, phosphorylation of the RelA subunit of NF- κ B by IKK α may lead to the degradation of promoter-bound RelA, which results in control of inflammation. IKK α activation could therefore both induce and limit NF- κ B activity. In addition, NF- κ B activation involving p50/p50 dimers, which lack transcriptional activation domains, has been observed during resolution of inflammation (Lawrence *et al*, 2001, 2005; Han and Ulevitch, 2005).

2.3. NF- κ B, cell proliferation and apoptosis

Suppression of NF- κ B in different cancer cell lines and in *in vivo* cancer models inhibits proliferation, causes cell cycle arrest, and leads to apoptosis, demonstrating the crucial role of NF- κ B in cell proliferation and survival (Aggarwal, 2004).

A number of cytokines regulated by NF- κ B act as growth factors in various types of cancer. IL-1 β , TNF, IL-6 and EGF have been reported to act as growth factors in acute myeloid leukemia, Hodgkin's lymphoma, cutaneous T cell lymphoma, gliomas and multiple myeloma, among others. IL-1, TNF and EGF stimulate cell proliferation through activation of NF- κ B. Inhibition of NF- κ B activation can reduce cyclin D1 activity, a positive regulator of G1-to-

S-phase progression, leading to delayed cell cycle progression (Yamamoto and Gaynor, 2001; Aggarwal, 2004).

Mice embryos lacking RelA die due to massive apoptosis of the liver, secondary to the proapoptotic effects of TNF (Beg *et al*, 1995).

NF- κ B directly activates several antiapoptotic genes such as cellular inhibitors of apoptosis (c-IAP1, c-IAP2 and IXAP), TNF receptor-associated factors (TRAF1 and TRAF2) and the Bcl-2 homolog A1/Bfl-1. Cellular inhibitors of apoptosis block activation of caspase-8, an initiator protease, involved at an early stage in stimulating the apoptotic pathway. NF- κ B directly induces expression of A1/Bfl-1 by binding to specific sites in its promoter, which prevents cytochrome *c* release from mitochondria and activation of caspase-3 in the B lymphocyte, playing an important role in the survival of this cell type (Grumont *et al*, 1998; Yamamoto and Gaynor, 2001).

In T lymphocytes, NF- κ B may stimulate expression of the Fas ligand, activating induced cell death, which suggests that NF- κ B proteins have different effects on apoptosis in different cell types (Kasibhatla *et al*, 1999; O'Connor *et al*, 2000).

2.4. NF- κ B, invasion and angiogenesis

Expression of proteins involved in cellular invasion and angiogenesis can be upregulated by NF- κ B transcriptional activation.

MMPs, uPA and tissue plasminogen activator (tPA) proteases, responsible for cellular invasion, have been found to be positively

regulated by NF- κ B. MMP-1, -2, -3, -9 and -13 expression is modulated by NF- κ B in different cell types (Farina *et al*, 1999; Baldwin, 2001; Andreakos *et al*, 2003). The uPA promoter contains an NF- κ B binding site that directly induces uPA expression by RelA, and uPA is transcriptionally activated by IL-1 and TNF α through induction of NF- κ B activity (Novak *et al*, 1991; Wang *et al*, 1999b; Aggarwal, 2004).

Inflammatory cells such as macrophages and neutrophils produce growth factors and chemokines known to be regulators of angiogenesis, including MCP-1, IL-8, TNF and VEGF, which are upregulated by NF- κ B activation (Chilov *et al*, 1997; Kiriakidis *et al*, 2003).

2.5. NF- κ B, estrogens and progesterone

ER and PR have been found to engage in antagonistic cross-talk with NF- κ B in different cell types, but these interactions appear to be complex and probably specific to cell type (Stein and Yang, 1995; Kalkhoven *et al*, 1996; McKay and Cidlowski, 1998, 1999; Kalaitzidis and Gilmore, 2005). In osteoblasts, NF- κ B interacts with ER, resulting in mutual repression. This interaction reduces NF- κ B binding to its promoter (as shown for IL-6 gene regulation) as well as ER binding to promoters with ER binding sites. This interaction thus leads to reduced transcriptional activity of NF- κ B- and ER-dependent promoters (Stein and Yang, 1995). Several mechanisms can explain the interaction

between ER and NF- κ B: 1) repression of NF- κ B-DNA binding by physical association with ER; 2) regulation of I κ B expression by estrogen; 3) repression of NF- κ B signaling by ER-dependent increased expression of PR (McKay and Cidlowski, 1999).

A study by Kalkhoven *et al* (1996) showed mutual repression between hormone-activated PR (A and B isoforms) and RelA in HeLa cells (epithelial cells), COS-1 cells (kidney fibroblast cells) and T47D cells (human breast tumor cell lines) *in vitro*. This mutual repression could have resulted from direct physical interaction between the two proteins, forming inactive complexes capable of binding to DNA, but unable to interact with essential co-factors or the basal transcriptional machinery.

McKay and Cidlowski (1998), working on COS-1 cell cultures, evaluated the effect of increasing amounts of RelA on PR-B-, AR- and ER-mediated transactivation. They proved that these steroid hormone receptors are inhibited by RelA in a similar way to glucocorticoid receptor (GR), theoretically concluding that a similar direct physical interaction or competition for co-factors underlies transrepression in each case. Contrary to the previously cited findings of Stein and Yang (1995) and Kalkhoven *et al* (1996), PR-B and ER had no effect on RelA activation in this study, failing to show any mutual antagonism between these molecules. The authors explained these discrepancies by the existence of cell type-specific differences in steroid receptor/RelA interactions, which is consistent with the hypothesis

that co-factor competition is involved in NF- κ B/steroid receptor antagonism.

2.6. NF- κ B and oxidative stress

ROS have also been implicated in the activation of NF- κ B (Schreck *et al*, 1991; Flohé *et al*, 1997). Basically, three observations have been made involving cellular oxidative signaling in NF- κ B activation: 1) antioxidants such as pyrrolidinedithiocarbamic acid (PDTC) and N-acetylcysteine (NAC) inhibit activation of NF- κ B; 2) administration of hydrogen peroxide (H_2O_2) to certain cells stimulates activation of NF- κ B; 3) most agents activating NF- κ B initiate formation of ROS. In murine macrophages, mitochondrial ROS are required for hypoxic activation of NF- κ B and TNF- α gene transcription, but not for LPS-dependent activation of NF- κ B (Chandel *et al*, 2000; Hayakawa *et al*, 2003).

Nonetheless, the contribution of redox regulation to NF- κ B activation is controversial because of conflicting findings. Activation of NF- κ B by H_2O_2 is highly cell type-dependent (Anderson *et al*, 1994; Bowie *et al*, 1997; Li and Karin, 1999; Korn *et al*, 2001). Thus, NF- κ B activation does not appear to be a universal response to oxidative stress. Hayakawa *et al* (2003) showed that both NAC and PDTC inhibit NF- κ B activation independently of antioxidative function in epithelial and fibroblast cell lines respectively, and that endogenously produced ROS do not lead to NF- κ B activation in epithelial cell lines.

ROS are important in mediating cross-talk between c-Jun NH₂-terminal kinase (JNK) and TNF-dependent NF- κ B activation. TNF receptor-1 ligation activates NF- κ B and JNK signaling, two pathways with opposing biological roles. JNK activation promotes apoptosis via the mitochondrial-dependent pathway, and NF- κ B activation promotes cell survival by upregulating expression of antiapoptotic members of the Bcl2 family and caspase inhibitors, and by down-regulating JNK activation. Indeed, some studies have reported that NF- κ B down-regulates JNK activation by suppressing TNF-induced ROS accumulation. TNF-dependent NF- κ B activation can lead to expression of antioxidant enzymes, which may explain the more efficient ROS clearance (Bubici *et al*, 2004; Papa *et al*, 2004; Sasazuki *et al*, 2004; Gloire *et al*, 2006).

3. The NF- κ B pathway in endometriosis *in vitro*

3.1. NF- κ B and endometrium

The first study to investigate NF- κ B in human endometrium was published in 2000 (Laird *et al*). Human endometrial epithelial and stromal cells showed expression of the RelA subunit of NF- κ B, as evaluated by immunohistochemical staining. RelA expression was stable in stromal cells during the menstrual cycle, with a slight increase during the periovulatory phase. In epithelial cells, RelA staining varied greatly throughout the cycle: in the proliferative phase, RelA expression was absent or very weak, but in the secretory phase, staining was more extensive, reaching maximum levels at the time of implantation and decreasing again at the end of the cycle. In this study, addition of the NF- κ B nuclear translocation inhibitor SN-50 and the proteasome inhibitor MG132 to endometrial epithelial cell cultures inhibited IL-1 α and TNF- α stimulated IL-6 and leukemia inhibitory factor (LIF) production, supporting the hypothesis that NF- κ B regulates IL-1 α - and TNF α -induced expression of LIF and IL-6 in endometrial epithelial cells.

King *et al* (2001) detected RNA expression of I κ B α , IKK α , IKK β , IKK γ and NF- κ B-inducing kinase (NIK) in human endometrium at different phases of the cycle. IKK α and NIK were immunolocalized mainly in the glandular epithelium and endothelium of the

endometrium. I κ B α mRNA levels were found to be significantly greater in perimenstrual endometrium. In this study, I κ B α was positively regulated by P in culture of endometrial epithelial cells. The authors postulated that P withdrawal at the end of the menstrual cycle activates NF- κ B, which could be responsible for the increase in I κ B α expression during the perimenstrual period. Both the p50 subunit of NF- κ B and I κ B β have been found to be expressed in endometrial epithelial and stromal cells, as shown by immunohistochemical analyses (Page *et al*, 2002).

Slight activation of the NF- κ B pathway has been demonstrated in non-stimulated endometrial epithelial and stromal cell cultures (Tamura *et al*, 2002a; Han and Sidell, 2003).

Expression of the proinflammatory mediators RANTES, COX-2 and MIF was shown to be upregulated by NF- κ B activation in IL-1 β - or TNF- α -stimulated endometrial stromal cells *in vitro* (Tamura *et al*, 2002b; Cao *et al*, 2005, 2006). The non-specific NF- κ B inhibitor, sulindac, was tested in endometrial stromal cell cultures, showing a reduction in TNF- α -induced RANTES expression as a result of decreased NF- κ B activation (Wieser *et al*, 2005). The positive modulation of RANTES, COX-2 and MIF supports NF- κ B as a proinflammatory transcription factor in endometrial stromal cells. Moreover, NF- κ B plays a role in cell growth, angiogenesis and tissue remodelling, sustained by the upregulation of MIF in these cells.

Concerning oxidative stress in endometrial stromal cell cultures, NF- κ B has been found to induce expression of manganese superoxide dismutase (Mn-SOD) in response to TNF- α . Mn-SOD protects cells by scavenging superoxide radicals. The involvement of NF- κ B in Mn-SOD induction by TNF- α may be considered a self-defense mechanism against TNF- α -mediated oxidative stress (Sugino *et al*, 2002). E and P withdrawal in endometrial stromal cells *in vitro* stimulates COX-2 and prostaglandin F2 α expression through ROS-induced NF- κ B activation, suggesting a possible mechanism for menstruation (Sugino *et al*, 2004).

A controversial study on endometrial epithelial cell lines showed that the antiprogestin mifepristone (RU486) inhibits endometrial epithelial cell growth and induces apoptosis through stimulation of NF- κ B (Han and Sidell, 2003).

3.2. Induced NF- κ B activity in endometriotic stromal cell cultures

NF- κ B-dependent transcriptional activation of proinflammatory genes, such as IL-1 or TNF- α , may provide positive feedback to the pathway, self-perpetuating the inflammatory response and providing possible mechanisms to resist apoptosis and stimulate cell proliferation in endometriotic lesions (Guo, 2007). This hypothesis is based on knowledge of the NF- κ B pathway and the inflammatory response in normal endometrium, ectopic endometrial stromal cells in culture and other type of cells or pathologies. A few *in vitro* studies have shown

that NF- κ B can be activated in endometriotic stromal cells by known inducers of the canonical NF- κ B pathway, such as IL-1 β , TNF- α or LPS (Lebovic *et al*, 2001; Sakamoto *et al*, 2003; Iba *et al*, 2004). The chemoattractant chemokine RANTES was up-regulated by IL-1 β via NF- κ B activation in endometriotic stromal cells, possibly producing a feed-forward regulatory loop and increasing macrophage recruitment (Lebovic *et al*, 2001). IL-8 and IL-6 expression was induced by TNF- α , probably through NF- κ B activation in endometriotic stromal cells (Sakamoto *et al*, 2003; Yamauchi *et al*, 2004). LPS-induced IL-8 production and endometriotic stromal cell proliferation were reduced by an NF- κ B inhibitor *in vitro* (Iba *et al*, 2004). Sulindac, thalidomide, GnRH-a, P and progestational compounds have all been tested in endometriotic stromal cell cultures, showing a reduction in RANTES and TNF- α -induced IL-8 expression as a result of decreased NF- κ B activation (Sakamoto *et al*, 2003; Horie *et al*, 2005; Wieser *et al*, 2005; Yagyu *et al*, 2005). Non-stimulated endometriotic stromal cells *in vitro* have shown slight NF- κ B activation (Horie *et al*, 2005; Wieser *et al*, 2005).

To date, there is no direct evidence of constitutive NF- κ B-DNA binding activity in endometriosis *in vivo*. Furthermore, the involvement of the NF- κ B pathway in the development of endometriotic lesions and the inflammatory response in endometriosis *in vivo*, and the effect of NF- κ B activation or inactivation on cell

proliferation and apoptosis in endometriosis, have not been previously studied.

Chapter II: Objective and experimental approach

1. Background

In the introduction, the multifactorial character of the etiopathogenesis of peritoneal endometriosis was explained, highlighting local immune and proinflammatory alterations in the pelvic cavity and the importance of the capacity of ectopic endometrial tissue to survive, adhere, invade, generate its own blood supply, proliferate and resist apoptosis. The transcription factor NF- κ B regulates the transcription of multiple genes involved in all the previously mentioned cell processes, playing a central role in the etiology of a number of inflammatory diseases and cancer. *In vitro* studies on endometriotic stromal cells suggest that activation of the NF- κ B pathway may play a role in the endometriosis-associated inflammatory response and cell proliferation. Since *in vitro* conditions may differ from *in vivo* conditions, and regulation of the NF- κ B pathway and processes under the control of NF- κ B transcriptional activity have proved to be cell type-specific, further research is required to study the involvement and role of the NF- κ B pathway in peritoneal endometriosis *in vivo*.

2. Objective

The aim of this study was to investigate the involvement and role of the NF- κ B pathway in endometriosis *in vivo*. Two experimental models were used to obtain additional and complementary information:

- 1) Examination of biopsies collected from patients.
- 2) Investigation of endometriosis induced in a nude mouse model.

During the first part of the study, the NF- κ B activation status and the inflammatory response by assessment of ICAM-1 expression were evaluated in biopsies of peritoneal endometriotic lesions from women.

The second part was designed to evaluate the impact of two different NF- κ B inhibitors on endometriosis development in an *in vivo* murine experimental model. This model allowed investigation of processes regulated by NF- κ B in endometriosis.

2.1. Part 1: NF- κ B activation and inflammatory response in biopsies of peritoneal endometriotic lesions from women

The aim of the first part of this study was to determine whether NF- κ B is constitutively activated in peritoneal endometriosis in women.

Due to the different evolutionary and inflammatory status of red and black peritoneal endometriotic implants, NF- κ B-DNA binding activity

and expression of the NF- κ B inhibitory protein I κ B α were investigated in these lesions.

Since the promoter region of the ICAM-1 gene contains putative recognition sequences for NF- κ B, and ICAM-1 mRNA and protein have been found to be enhanced in endometriotic cell cultures, expression of this protein was evaluated as an NF- κ B-regulated inflammatory marker in peritoneal endometriotic lesions.

The p65 and p50 subunits of active NF- κ B dimers were also evaluated in endometriotic lesions to gain some insight into the pathways implicated in NF- κ B activation in peritoneal endometriosis.

With these ends in mind, several laboratory techniques were set up during the study period. The first challenge was to develop protein extraction procedures from small biopsies (weighing between 5 and 100 mg). Electrophoretic mobility shift assays (EMSAs) were performed in collaboration with the gastroenterology unit of the UCL. NF- κ B (p65)-DNA binding activity immunodetection assays (TransAM[®] kit) and Western blotting using protein extracts from endometrial and peritoneal endometriosis samples were applied for the first time in our laboratory.

2.2. Part 2: testing two different NF- κ B inhibitors in an *in vivo* experimental model of endometriosis

The second part of this study was designed to investigate the involvement of the NF- κ B pathway in the development of endometriotic lesions and NF- κ B regulation of inflammation, cell proliferation and apoptosis in endometriotic lesions in an *in vivo* experimental model of endometriosis. To this end, the impact of two NF- κ B inhibitors, BAY 11-7085 and SN-50, was tested on the development of endometriosis induced in nude mice. BAY 11-7085 is an inhibitor of I κ B α phosphorylation that prevents the release of NF- κ B into the cytoplasm, its translocation to the nucleus and binding to DNA. SN-50 is a peptide carrying a functional cargo in the form of the nuclear localization sequence of NF- κ B p50, which specifically inhibits NF- κ B nuclear translocation (Figure 10).

In order to reliably quantify the impact of NF- κ B inhibitors on the initial development of endometriotic lesions, a new *in vivo* model of endometriosis was developed in our laboratory. This novel nude mouse model induces endometriotic lesion formation through injection of human menstrual endometrium into the pelvic cavity of nude mice. It allows more precise and reliable identification and quantification of lesions in mice than earlier models. A new surgical procedure reducing the risk of possible bias resulting from scar inflammation was applied, and two new methods to quantify human

endometriotic lesions induced in nude mice were validated: fluorimetry and morphometry (Annex I, article 3).

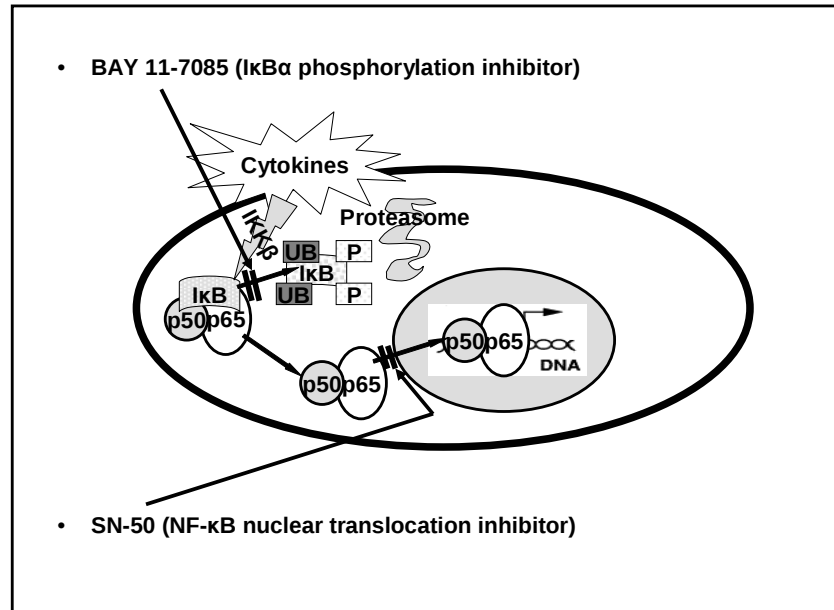


Figure 10: NF-κB inhibitors.

3. Experimental approach

3.1. Part 1: NF-κB activation and inflammatory response in biopsies of peritoneal endometriotic lesions from women

Peritoneal endometriotic lesions were obtained during laparoscopic surgery from women with endometriosis. Biopsies were transported on ice and immediately stored at -80°C until protein extraction.

Preliminary experiments were performed on endometrial tissue samples and endometriotic lesions to obtain adequate protein concentrations after protein extraction procedures. Inclusion of protease and phosphatase inhibitors in the cytoplasmic and nuclear extraction reagents was essential to preserve the samples. The impact of freezing on protein concentrations was also assessed.

Two methods were used to evaluate the NF- κ B-DNA binding activity of endometriotic lesions:

- 1) The classic EMSA method, which was validated to provide quantitative data.
- 2) The NF- κ B (p65)-DNA binding activity immunodetection assay (TransAM[®] kit), which further confirmed the EMSA results and provided additional information on concentrations of active p65-containing NF- κ B dimers in nuclear extracts of endometriotic lesions.

In the EMSAs performed (Figure 11), NF- κ B nucleoprotein-oligonucleotide complexes were quantified by optical densitometry (OD) of shifted bands relative to positive controls. This was validated as a quantitative method by preparing a dilution curve with nuclear extracts from one endometrial sample (see Figures 2C and 2D from Chapter III, article 1).

To detect the presence of p50 and p65 subunits of NF- κ B in nuclear extracts of endometriotic lesions, supershift assays were performed using antibodies against the p50 and p65 subunits of NF- κ B.

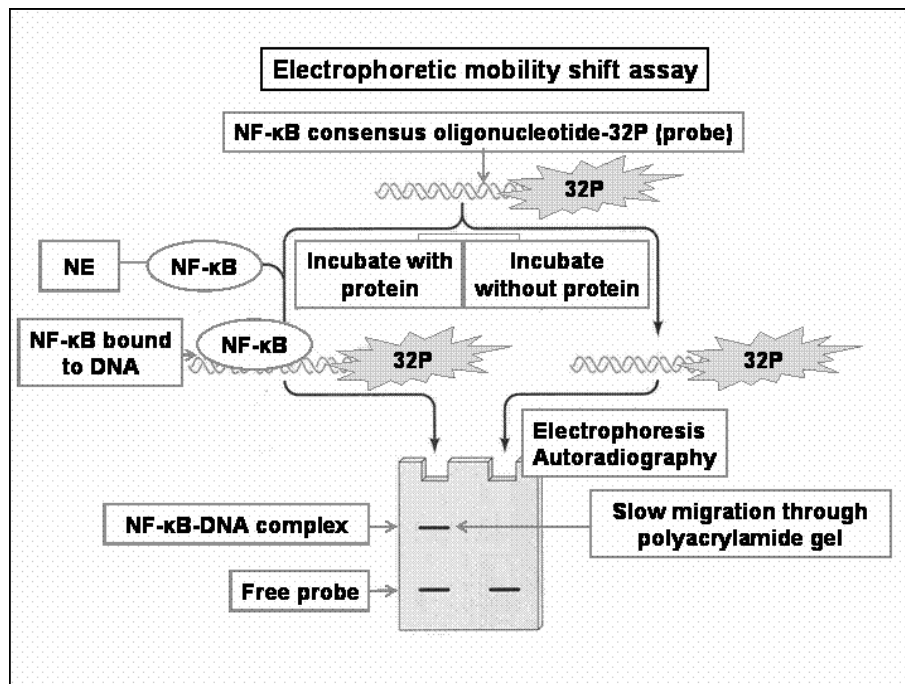


Figure 11: Electrophoretic mobility shift assay (EMSA). NE: nuclear extract.

The second method used to measure NF- κ B activation in endometriotic lesions was the NF- κ B (p65)-DNA binding activity immunodetection assay (Figure 12). This method is useful to measure concentrations of activated p65-containing NF- κ B dimers in nuclear extracts of endometriotic lesions. Stimulated Jurkat nuclear extracts were used as positive controls, and wild-type and mutated consensus

oligonucleotides to monitor the specificity of the assay. This technique was first set up using endometrial nuclear extracts.

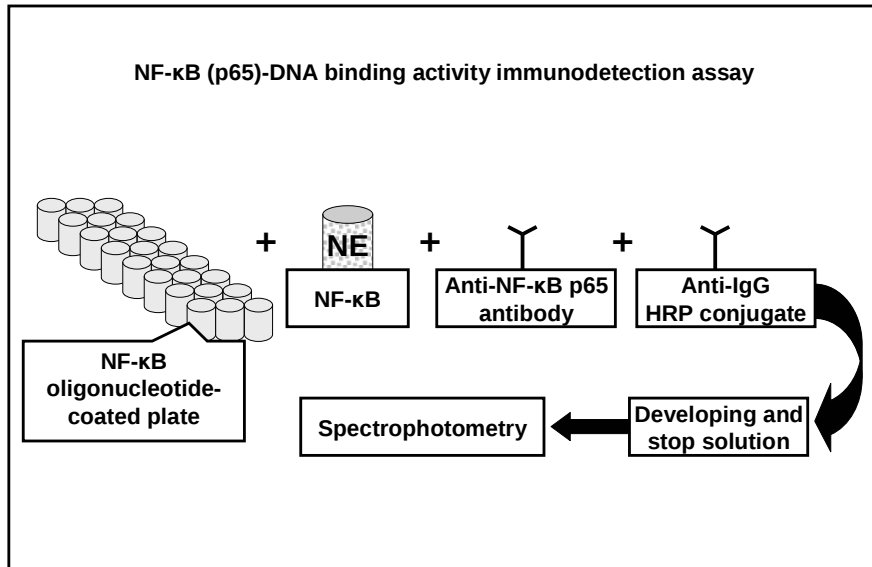


Figure 12: NF-κB (p65)-DNA binding activity immunodetection assay. NE: nuclear extract.

Western blot analyses were applied to determine IκBα and ICAM-1 expression in cytoplasmic extracts from peritoneal endometriotic lesions. Purified IκBα protein was used as a positive control for IκBα blots, and HeLa whole cell lysate as a positive control for ICAM-1 blots. The OD of the IκBα and ICAM-1 bands was measured and analyzed relative to the β-actin bands, used as internal controls. Western blot analysis was set up in our laboratory and validated as a

quantitative method by preparing dilution curves with cytoplasmic extracts from endometrial samples for all used antibodies.

3.2. Part 2: testing two different NF- κ B inhibitors in an *in vivo* experimental model of endometriosis

The first phase of this approach involved creating two new techniques to quantify the development of endometriotic lesions induced in the peritoneal cavity of nude mice: fluorimetry and morphometry. Fluorimetry measures the fluorescence of induced endometriotic lesions, human endometrium having been previously marked with a fluorescent tracker, and allows recovery and measurement of microscopic lesions. Morphometry or immunohistochemical morphometric analysis with CK22 and CD10 antibodies allows specific microscopic measurement of the surface area of all epithelial glands and stroma in the 2 largest sections of endometriosis-like lesions. CD10 is an immunohistochemical marker of endometrial stromal cells, while endometrial epithelial cells express CK22. These two techniques are described in full in Annex I (article 3).

In the second phase of this approach, endometriosis was induced in nude mice by intraperitoneal injection of human menstrual endometrium labeled with a fluorescent tracker. Treated mice were injected intraperitoneally either with vehicle (control groups), BAY 11-7085 or SN-50. Each experiment included one control mouse and

one treated mouse, both injected at the same time with one homogenized endometrial sample from one single patient (Figure 13).

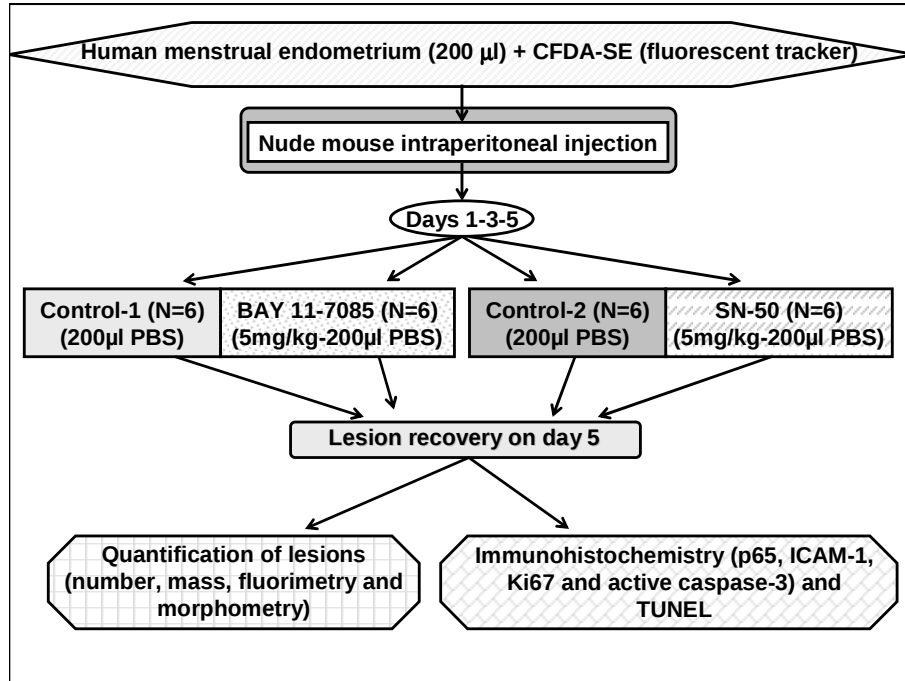


Figure 13: Experimental design: nude mouse model.

Endometriosis-like lesions were recovered, counted and quantified by fluorimetry, mass and morphometry. NF- κ B activation was assessed by immunolocalization of its p65 (RelA) subunit. To evaluate the downstream effects of the NF- κ B response, ICAM-1 expression was quantified by immunohistochemical analysis, ectopic endometrial cell proliferation was measured by Ki67 immunostaining, and apoptosis was quantified by active caspase-3 immunostaining and the TUNEL (TdT-mediated biotin-dUTP nick-end labeling) method. NF- κ B,

ICAM-1 and active caspase-3 immunostaining and TUNEL were first performed in endometrial samples, before being applied to endometriotic lesions.

Chapter III: Is the NF-κB pathway activated in peritoneal endometriosis in women?

1. Article 1: Nuclear factor-kappaB (NF-κB) is constitutively activated in peritoneal endometriosis

Nuclear factor-kappa B is constitutively activated in peritoneal endometriosis

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Running title: NF-κB and ICAM-1 in peritoneal endometriosis

Keywords: endometriosis / ICAM-1 / IkappaB / inflammation / NF-kappaB

Chapter IV: Involvement of NF-κB in the development of endometriotic lesions

1. Article 2: Agents blocking the nuclear factor-kappaB (NF-κB) pathway are effective inhibitors of endometriosis in an *in vivo* experimental model

Reinaldo González-Ramos¹, Anne Van Langendonck¹, Sylvie Defrère¹, Jean-Christophe Lousse¹, Marcel Mettlen², Alain Guillet³ and Jacques Donner¹. *Gynecol Obstet Invest*, *in press*.

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Short title: Nuclear factor-κB inhibition reduces endometriosis *in vivo*

Keywords: apoptosis / BAY 11-7085 / cell proliferation / endometriosis / inflammatory response / ICAM-1 / NF-kappaB inhibition / nude mouse model / SN-50

Chapter V: Conclusions

1. Constitutive activation of the NF- κ B pathway in peritoneal endometriosis

NF- κ B activation can be triggered by different stimuli *in vitro* or *in vivo* but, in most normal cells, NF- κ B is basally inactive. Constitutive or non-induced activation of the NF- κ B pathway occurs in B cells, some monocyte cell lines and in most tumor cell lines and tumor tissues derived from patients with different types of cancer (Rice and Ernst, 1993; Aggarwal, 2004). Constitutive NF- κ B activation therefore seems to be a pathological event in cells or tissues other than B cells and certain monocyte cell lines. In endometrium, however, the activation status of the NF- κ B pathway remains unclear. Existing data suggest that NF- κ B could be physiologically activated in normal endometrium during the implantation period and menstruation, but these studies have not shown constitutive NF- κ B-DNA binding activity in normal endometrium *in vivo* (Laird *et al*, 2000; King *et al*, 2001). *In vitro* studies have demonstrated slight constitutive activation of NF- κ B in non-stimulated endometrial epithelial and stromal cells, and in endometriotic stromal cells (Tamura *et al*, 2002a; Han and Sidell, 2003; Horie *et al*, 2005).

This is the first study to demonstrate constitutive *in vivo* activation of the NF- κ B pathway in peritoneal endometriotic lesions in women and in the very early stages of endometriotic lesion development in nude

mice. Furthermore, red endometriotic lesions show a significantly higher degree of NF- κ B pathway activation than black lesions (González Ramos *et al*, 2007a, 2007b). A recent study found higher expression of IL-1RI in red endometriotic lesions than black and white lesions (Lawson *et al*, 2007), which corroborates our findings, since this receptor is one of the main receptors activating the NF- κ B pathway. Unpublished data from our laboratory show increased NF- κ B activation in peritoneal macrophages in women with endometriosis (Lousse *et al*, submitted). Increased expression of IL-1RI and increased NF- κ B activation in peritoneal macrophages in endometriosis patients could be factors responsible for the increased constitutive NF- κ B activation and inflammatory response in red endometriotic lesions.

Analysis of the NF- κ B dimers involved in the constitutive activation of NF- κ B in endometriotic lesions revealed DNA-binding activity of p50/p65 and p50/p50 dimers. Concentrations of active p65-containing dimers were significantly higher in red endometriotic lesions than black lesions, which is important, since p50/p65 are transcriptionally active dimers, while the p50/p50 homodimer functions as a repressor as it lacks a transcription activation domain (Bonizzi and Karin, 2004). Moreover, these results, as well as analysis of published data, suggest that the canonical pathway could be the main activation pathway of NF- κ B in endometriotic lesions (González Ramos *et al*, 2007a).

The NF- κ B inhibitor, I κ B α , was found to be expressed in equal measure in red and black peritoneal endometriotic lesions (González Ramos *et al*, 2007a). NF- κ B regulation involves negative feedback inhibition through NF- κ B-directed synthesis of I κ B α (Chiao *et al*, 1994). I κ B α enters the nucleus, removes NF- κ B from the DNA, and then exports it to the cytoplasm, restoring the pool of inactive NF- κ B/I κ B α complexes (Arenzana-Seisdedos *et al*, 1997). In pre-B or mature B cells, there is a balanced state between the rates of degradation and synthesis of I κ B α proteins (Rice and Ernst, 1993), which could also occur in endometriotic cells.

In conclusion, this is the first study to show constitutive NF- κ B activation in peritoneal endometriotic lesions collected from women and during the initial development of endometriotic lesions in an animal model. Differential levels of NF- κ B-DNA binding activity have been established, for the first time, between red and black endometriotic lesions, providing more evidence on the distinct inflammatory status of these two types of peritoneal endometriotic lesions (Figure 14).

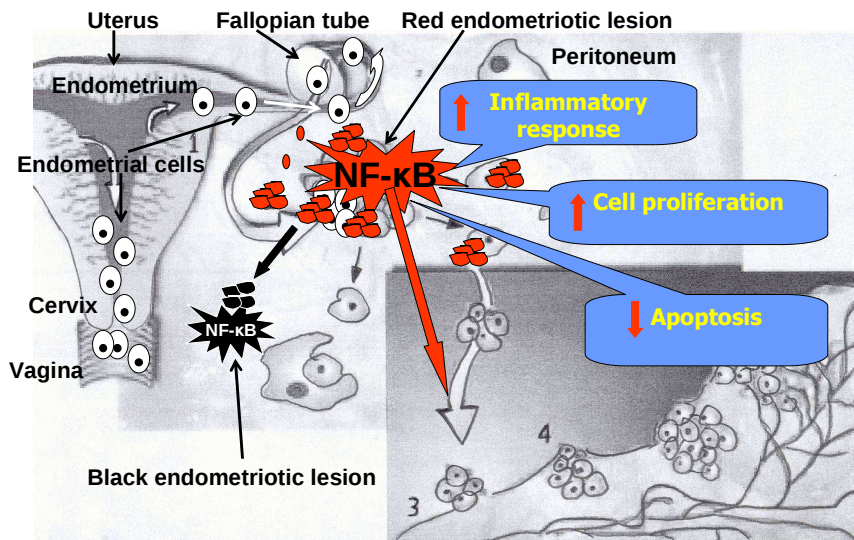


Figure 14: NF- κ B involvement in peritoneal endometriosis. NF- κ B is constitutively activated in peritoneal endometriosis. This activation is higher in active red endometriotic lesions than in black lesions. Activation of NF- κ B during the initial development of endometriotic lesions enhances the inflammatory response and cell proliferation of endometriotic lesions and inhibits apoptosis (adapted from Groothuis *et al*, 2005).

2. The NF- κ B pathway in the development of endometriosis

As detailed in the introduction, endometriosis is a multifactorial disease, with many cellular processes and biomolecules found to be altered, conditioning the establishment and development of peritoneal endometriotic lesions. Both experimental approaches applied in this study confirm the involvement of the NF- κ B pathway in the initial development of endometriotic lesions.

Since red endometriotic implants are considered to be the most active lesions and the first stage of peritoneal endometriosis (Nisolle and Donnez, 1997; Khan *et al*, 2004; Van Langendonckt *et al*, 2004), and as they show higher NF- κ B activation than black lesions (González Ramos *et al*, 2007a), it is highly plausible that NF- κ B activation plays an important role in the early stages of endometriotic lesion establishment (Figure 14). Furthermore, as described in Chapter IV (article 2) of this thesis, inhibition of NF- κ B activation was shown to reduce the initial development of endometriotic lesions of human origin induced in an *in vivo* experimental model, demonstrating that the NF- κ B pathway plays an important role in the early development of such lesions *in vivo* (González Ramos *et al*, 2007b).

3. Processes regulated by the NF- κ B pathway in endometriotic lesions

All cell processes involved in the establishment and development of peritoneal endometriotic lesions have been found to be regulated by the NF- κ B pathway in many other cell types. These cell processes include adhesion, invasion, inflammation, angiogenesis, proliferation and apoptosis. However, in endometriosis, only a few studies have shown regulation of some inflammatory factors and cell proliferation by NF- κ B *in vitro*.

Concerning the endometriosis-associated inflammatory response, our studies have shown higher NF- κ B activation and ICAM-1 expression

in red endometriotic lesions than black endometriotic lesions (González Ramos *et al*, 2007a). Since the promoter region of the ICAM-1 gene contains putative recognition sequences for NF- κ B (Chen *et al*, 2000), and NF- κ B inhibition reduces ICAM-1 expression of endometriotic lesions *in vivo* (González Ramos *et al*, 2007b), it is highly plausible that increased ICAM-1 expression results from the activation of NF- κ B in endometriotic lesions.

Two important conclusions can be drawn from these results: 1) red endometriotic lesions show a stronger inflammatory response than black lesions, and 2) the inflammatory response observed in peritoneal endometriosis *in vivo* is at least partly caused by activation of the NF- κ B pathway. The first assumption is consistent with other studies that have found more extensive inflammatory reactions in red endometriotic lesions than black lesions (Kokorine *et al*, 1997; Kats *et al*, 2002; Khan *et al*, 2004). The second statement agrees with studies showing modulation of proinflammatory factors by NF- κ B in macrophages and endometrial stromal cells (Bondeson *et al*, 1999; Cao *et al*, 2006).

Two other processes theoretically regulated by NF- κ B in peritoneal endometriotic lesions are cell proliferation and apoptosis. Both were studied in endometriotic lesions induced in nude mice and both were found to be regulated by NF- κ B, since the administration of NF- κ B inhibitors decreased cell proliferation and induced apoptosis in endometriotic stromal and epithelial cells *in vivo*. This is the first time

NF- κ B activation has been shown to promote cell survival in early-stage endometriotic lesions *in vivo* by inducing cell proliferation and inhibiting apoptosis (González Ramos *et al*, 2007b). In addition, stronger activation of the NF- κ B pathway in red endometriotic lesions than black lesions is in line with other studies showing higher mitotic and lower apoptotic activities in red lesions (Nisolle and Donnez, 1997; Khan *et al*, 2003; Fujino *et al*, 2006). In other cell types too, NF- κ B activation has been found to promote cell survival (Perkins, 2007).

Two key statements can therefore be made on the basis of these findings: 1) red endometriotic lesions are highly active in terms of inflammation, proliferation and resistance to apoptosis and 2) constitutive activation of the NF- κ B pathway may be responsible for the inflammatory response, cell proliferation and resistance to apoptosis observed in peritoneal endometriotic lesions (Figure 14).

4. NF- κ B inhibition in endometriotic lesions

Chapter IV (article 2) of this thesis shows that NF- κ B inhibition curtails the initial development of endometriotic lesions induced in nude mice by reducing the inflammatory reaction, cell proliferation and inducing apoptosis of endometriotic lesions (Figure 15).

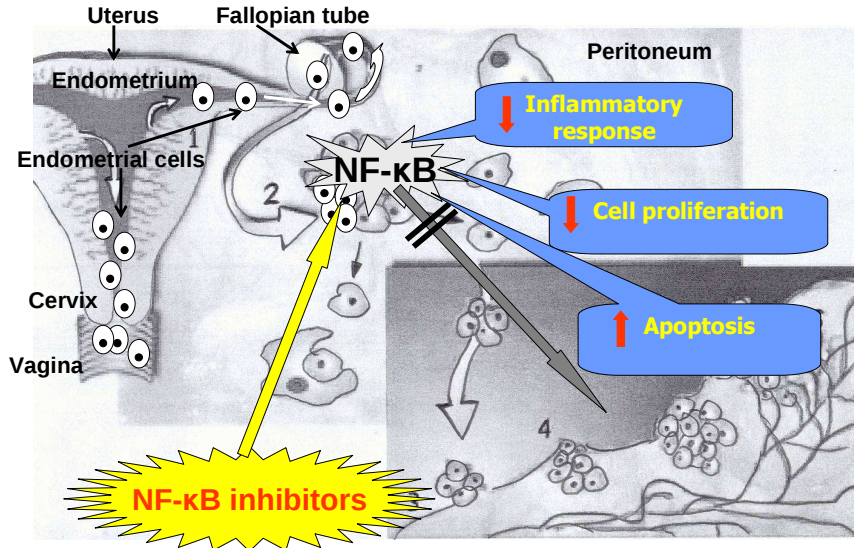


Figure 15: NF- κ B inhibition reduces the initial development of endometriotic lesions by inhibiting the inflammatory response and cell proliferation, and inducing apoptosis of endometriotic lesions (adapted from Groothuis *et al*, 2005).

Other studies have shown a reduction in endometriotic lesion development using different hormonal therapies (aromatase inhibitors, GnRH-a, progestogens, etc), anti-inflammatory drugs (selective COX-2 inhibitors, TNF- α inhibitors, PPAR- γ agonists), anti-MMPs and antiangiogenic agents (Bruner *et al*, 1997; Falconer *et al*, 2006; Mihalyi *et al*, 2006). PR has been shown to engage in antagonistic crosstalk with NF- κ B, COX-2 and MMPs can be upregulated by NF- κ B, while TNF- α is a well known inducer of the NF- κ B pathway and activation of NF- κ B upregulates the transcription of TNF- α .

(Kalkhoven *et al*, 1996; Farina *et al*, 1999; Hoffmann and Baltimore, 2006). In this study, we targeted the transduction pathway itself instead of one of the stimuli activating it or one of the NF- κ B-induced molecules, which is likely to affect a broader range of processes. The NF- κ B pathway therefore looks to be a promising therapeutic target for endometriosis.

Chapter VI: Perspectives

1. Is constitutive NF- κ B activation a primary event in the development of endometriosis?

More information is needed to confirm that NF- κ B activation is a primary event in the initial development of peritoneal endometriosis. Indeed, if activation of the NF- κ B pathway is responsible for the establishment of peritoneal endometriotic lesions, since the initial step in the development of these lesions occurs during menstruation, menstrual endometrium from patients with endometriosis should show higher constitutive NF- κ B activation than menstrual endometrium from control women without endometriosis. Experiments performed in our laboratory have revealed different degrees of constitutive activation of the NF- κ B pathway in different endometrial samples at different times of the menstrual cycle, but mostly in patients with endometriosis (unpublished data), and there are no studies comparing NF- κ B activation levels in endometrium from patients with and without endometriosis. Further research is therefore needed to characterize NF- κ B activation status more precisely, especially during menstruation, in endometrial samples from both control women without endometriosis and patients with endometriosis.

Future studies should also consider investigating the role of the NF- κ B pathway in cell processes involved in the initial development of

endometriotic lesions that were not evaluated in this thesis. These processes include cell adhesion, invasion and angiogenesis.

2. Which stimuli activate the NF- κ B pathway in endometriosis?

Many theories can be proposed to explain the constitutive activation of the NF- κ B pathway in peritoneal endometriosis. As explained in the introduction, the NF- κ B pathway is intricately regulated. Numerous mechanisms, involving multiple cytokines, enzymes and protein kinases, can activate NF- κ B and different activated NF- κ B dimers can trigger different transcriptional activities. Thus, the constitutive activation of NF- κ B observed in endometriosis could originate from intrinsic alterations to the pathway, or external stimuli activating it. Both hypotheses are appealing and plausible. Intrinsic alterations to the pathway may be explained by genetic or epigenetic changes, which could account for the hereditary component of endometriosis. A recent study showing higher expression of IL-1RI in red endometriotic lesions than black endometriotic lesions (Lawson *et al*, 2007) provides a possible explanation for the constitutive activation of the NF- κ B pathway in endometriosis. External stimuli activating the pathway may include hypoxia, oxidative stress and proinflammatory cytokines. Unpublished data from our laboratory show increased NF- κ B activation in macrophages in the pelvic peritoneal fluid of women with endometriosis, which could explain the increased levels of cytokines observed in endometriosis and the

constitutive NF- κ B activation of peritoneal endometriosis (Lousse *et al*, submitted). All these premises could lay the ground for new studies to better understand the mechanisms responsible for endometriosis development.

3. Which pathways are implicated in NF- κ B activation in endometriosis?

As mentioned in Chapter III, the canonical pathway of NF- κ B appears to be the main pathway activating NF- κ B in peritoneal endometriosis. Since activation of the canonical pathway is dependent on IKK β activation, this kinase warrants investigation as a potential target for new endometriosis therapies. However, activation of atypical NF- κ B pathways cannot be excluded in endometriotic lesions, since such pathways have also been shown to activate p50/p65 dimers in response to other kinds of stimuli, such as ROS, hypoxia-reoxygenation and genotoxic stress, but they have not been studied in the context of endometriosis.

It would therefore be interesting to study the activity of IKK β in endometriosis and to test IKK β inhibitors on the development of endometriotic lesions. Atypical NF- κ B activation pathways and the stimuli involved in activating them also need to be evaluated in normal and ectopic endometrial cells *in vitro* and *in vivo*.

4. The NF- κ B pathway as a novel therapeutic target for endometriosis

The central role that NF- κ B inhibitors play in curtailing the development of endometriotic lesions by reducing inflammation and cell proliferation and inducing apoptosis has been clearly established in this thesis. New efforts should now focus on testing NF- κ B inhibitors in more relevant models of endometriosis, such as the baboon or macaque. Since NF- κ B is involved in a wide range of important cell processes, the clinical application of NF- κ B inhibitors is not possible at this stage, and systemic use of this type of drug could well be dangerous. Further research is needed to evaluate the safety and side effects of these drugs before they can be considered suitable for use in humans. For these reasons, local treatments inhibiting NF- κ B look more attractive and are currently being evaluated by our group in one of the collaboration projects with the Université de Liège.

Annex I

1. Article 3: Quantification of endometriotic lesions in a murine model by fluorimetric and morphometric analyses

Quantification of endometriotic lesions in a murine model by fluorimetric and morphometric analyses

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Running title: Quantification of endometriosis in mice

Keywords: CFDA, SE / endometriosis / lesion quantification / menstrual endometrium / nude mouse model

Annex II

1. Publications

First author

González Ramos R, Donnez J, Defrère S, Leclercq I, Squifflet J, Lousse JC, Van Langendonck A (2007) Nuclear factor-kappaB (NF-κB) is constitutively activated in peritoneal endometriosis. *Mol Hum Reprod*, in press, advance access on line.

González Ramos R, Van Langendonck A, Defrère S, Lousse JC, Mettlen M, Donnez J (2007) Agents blocking the nuclear factor-kappaB (NF-κB) pathway are effective inhibitors of endometriosis in an *in vivo* experimental model. *Gynecol Obstet Invest*, in press.

Co-author

Defrère S, Van Langendonck A, González Ramos R, Jouret M, Mettlen M, Donnez J (2006) Quantification of endometriotic lesions in a murine model by fluorimetric and morphometric analyses. *Hum Reprod* 21,810-817.

Defrère S, Van Langendonck A, Vaesen S, Jouret M, González Ramos R, Gonzalez D, Donnez J (2006) Iron overload enhances epithelial cell proliferation in endometriotic lesions induced in a murine model. *Hum Reprod* 21,2810-2816.

Defrère S, Van Langendonck A, Moulin P, Befahy P, González Ramos R, Lousse JC, Donnez J (2007) Expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in human endometrial stromal and epithelial cells is regulated by interferon-gamma (IFN-γ) but not iron. *Gynecol Obstet Invest*, accepted.

Lousse JC, Van Langendonck A, González Ramos R, Defrère S, Renkin E, Donnez J. Increased activation of nuclear factor-kappaB (NF-κB) in isolated peritoneal macrophages of patients with endometriosis. *Fertil Steril*, submitted.

2. Communications

Effect of NF-kB inhibition on the development of endometriosis in a nude mouse model. González Ramos R, Van Langendonckt A, Defrère S, Mettlen M, Donnez J. *9th World Congress on Endometriosis – September 2005, Maastricht*.

Effect of NF-kB inhibition on the development of endometriosis in a nude mouse model. González Ramos R, Van Langendonckt A, Defrère S, Mettlen M, Donnez J. *Signal Transduction Pathways as therapeutic target-Cell Signaling World 2006-Luxembourg*.

Involvement of iron in the development of pelvic endometriotic lesions induced in a murine model. Defrère S, Van Langendonckt A, Vaesen S, Jouret M, González Ramos R, González D, Donnez J. *9th World Congress on Endometriosis-September 2005, Maastricht*.

Fluorimetric and morphometric quantification of endometriotic lesions in a murine model. Defrère S, Van Langendonckt A, González Ramos R, Jouret M, Mettlen M, Donnez J. *9th World Congress on Endometriosis-September 2005, Maastricht*.

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