

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 Unpublished data from our laboratory show increased NF- κ B activation in peritoneal macrophages in women with endometriosis (Lousse *et al*, submitted).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).

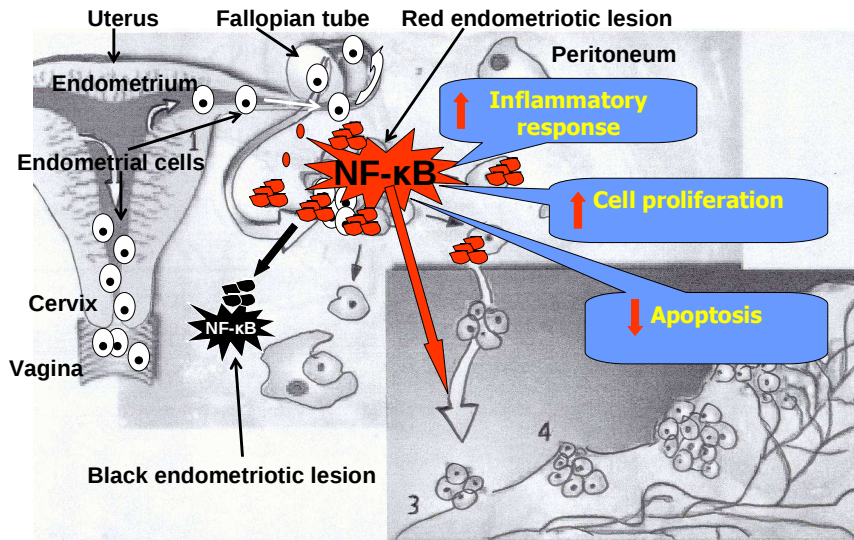


Figure 14: NF-κB involvement in peritoneal endometriosis. NF-κB is constitutively activated in peritoneal endometriosis. This activation is higher in active red endometriotic lesions than in black lesions. Activation of NF-κB during the initial development of endometriotic lesions enhances the inflammatory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

1. The NF-κB 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-κB 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).

2. 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).

3. 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).

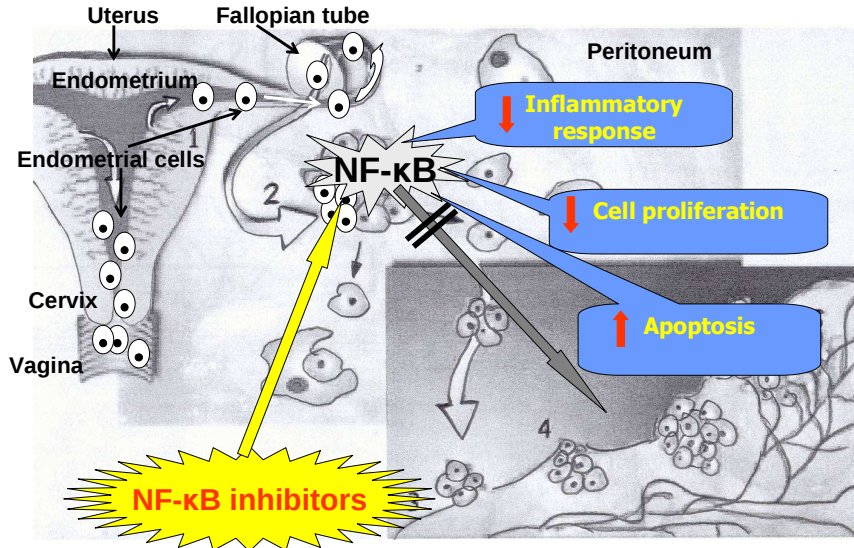


Figure 15: NF- κ B inhibition reduces the initial development of endometriotic lesions by inhibiting the inflammatory response and cell proliferation, and inducing apoptosis of endometriotic lesions (adapted from Groothuis *et al*, 2005).

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.