

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).

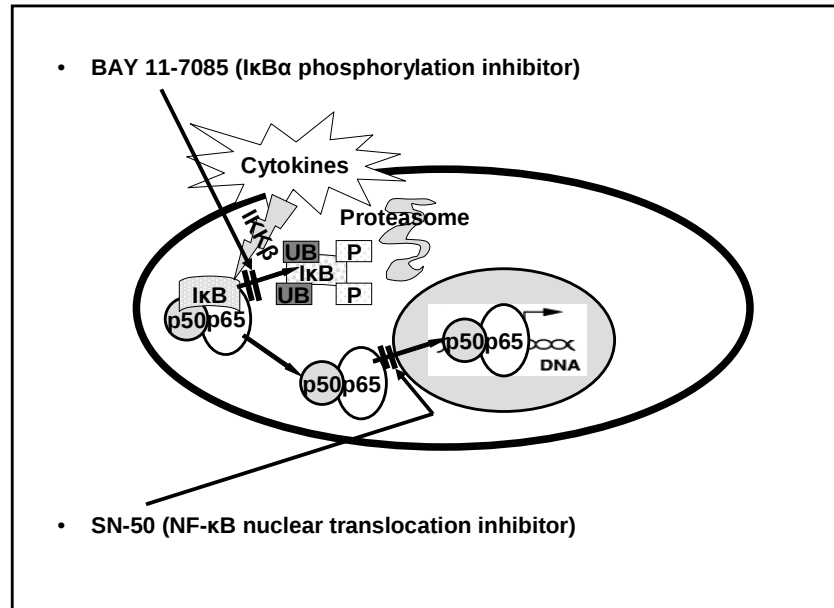


Figure 10: NF-κB inhibitors.

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.

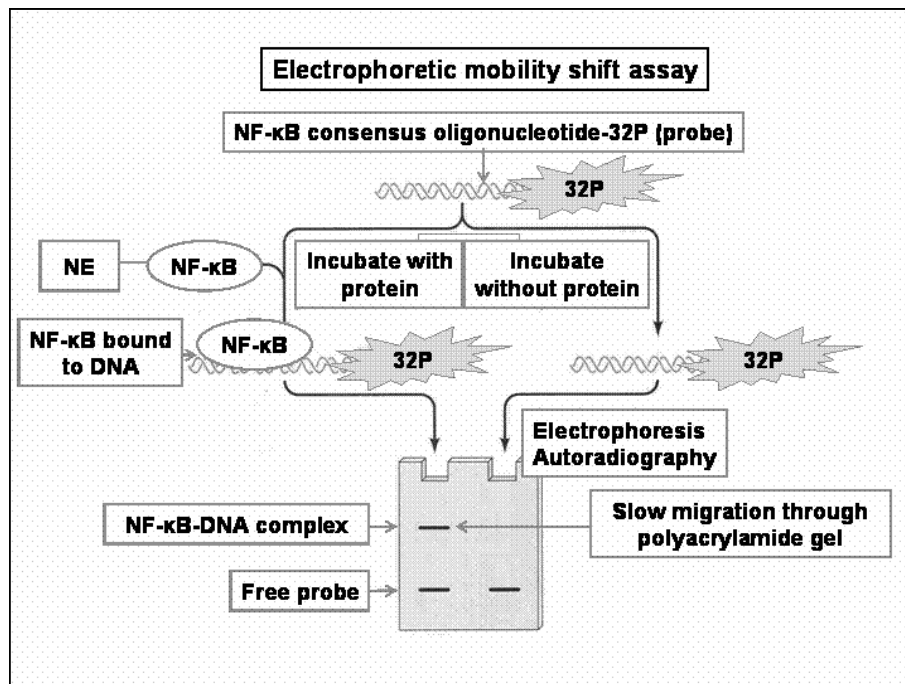


Figure 11: Electrophoretic mobility shift assay (EMSA). NE: nuclear extract.

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.

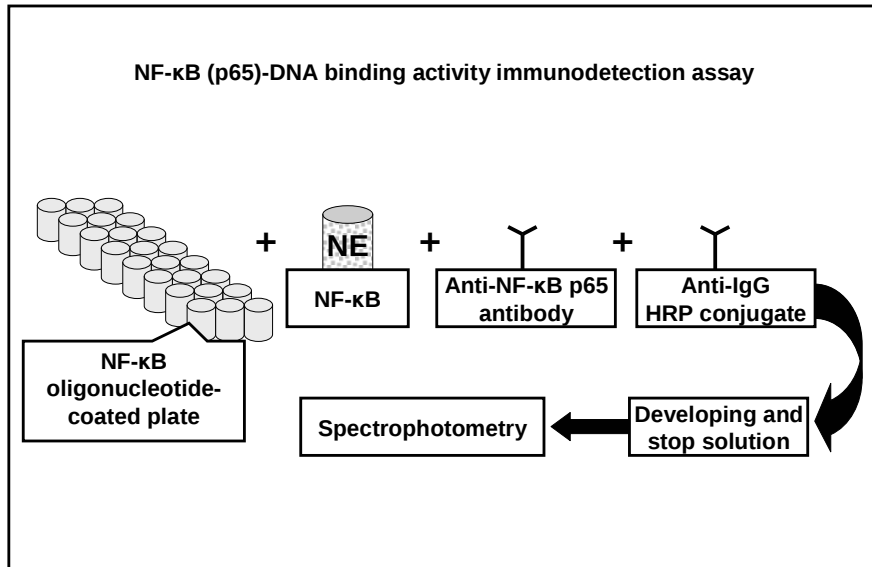


Figure 12: NF-κB (p65)-DNA binding activity immunodetection assay. NE: nuclear extract.

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).

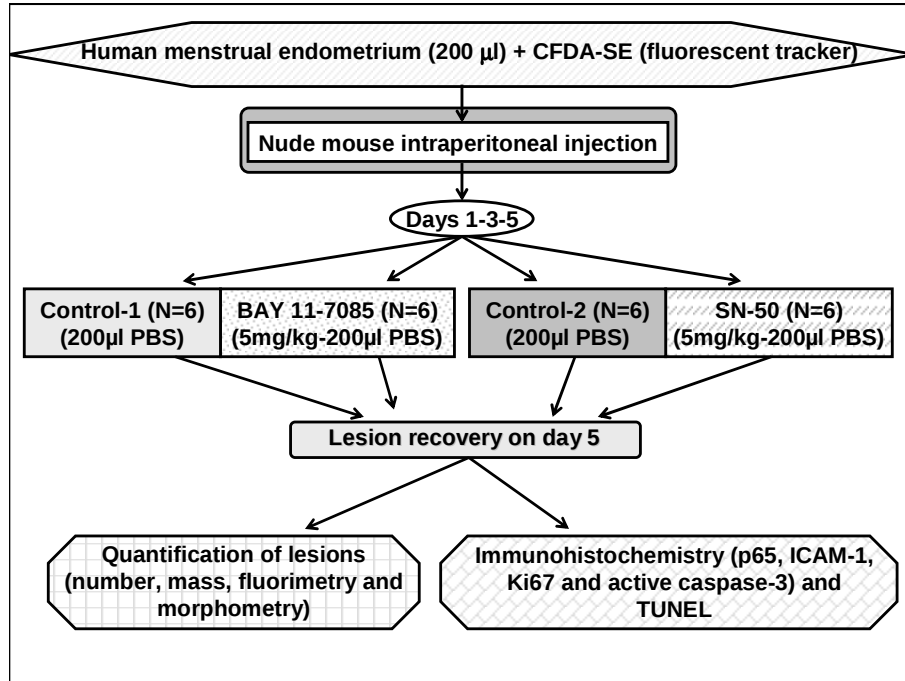


Figure 13: Experimental design: nude mouse model.

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.