Nuclear factor-kappa B is constitutively activated in peritoneal endometriosis

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Red (active), black and white endometriotic lesions are characteristic of peritoneal endometriosis. The transcription factor nuclear factor-kappa B (NF-κB) activates proinflammatory, proliferative and antiapoptotic genes in many cell types. To determine whether NF-κB is activated in peritoneal endometriosis in women, and further ascertain the differential inflammatory status of endometriotic implants, NF-κB activation and intercellular adhesion molecule (ICAM)-1 expression were investigated in peritoneal endometriotic lesions according to their type. Furthermore, p65 and p50 subunits of active NF-κB dimers were evaluated in endometriotic lesions to gain some insight into NF-κB-implicated pathways. Thirty-six biopsies of peritoneal endometriotic lesions were analyzed. Constitutive NF-κB activation, involving p65- and p50-containing dimers, was demonstrated in peritoneal endometriotic lesions by electrophoretic mobility shift assays and supershift analyses, as well as NF-κB (p65) DNA-binding activity immunodetection assays. NF-κB activation and ICAM-1 expression (evaluated by immunoblotting) were significantly higher in red lesions than black lesions, whereas IκBα (NF-κB inhibitory protein) expression was constant, as shown by western blot analysis. This is the first study to demonstrate constitutive NF-κB activation in peritoneal endometriosis in women. NF-κB activation and ICAM-1 expression in red lesions confirm the more extensive inflammatory pattern of these lesions compared with black lesions. The involvement of p50/p65 dimers in NF-κB activation suggests implication of the classic NF-κB activation pathway, making it an attractive therapeutic target in endometriosis.

Key words: endometriosis; ICAM-1; IκappaB; inflammation; NF-κB

Introduction

Endometriosis is a common gynecological pathology characterized by the presence of endometrial tissue outside the uterus. Infertility and pelvic pain are the most common problems encountered in patients with endometriosis, although it is often asymptomatic (Kennedy et al., 2005). In the pelvis, three different forms of endometriosis must be considered: peritoneal, ovarian and rectovaginal (Donnez et al., 1996; Nisolle and Donnez, 1997). Peritoneal lesions go through various stages and have a range of aspects, appearing as red, black and white lesions, 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 Langendonckt 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 (Nisolle and Donnez, 1997).

A number of etiopathogenic factors may explain the development, maintenance and progression of endometriotic lesions: (i) a proinflammatory and pro-oxidant environment has been implicated in the etiopathogenesis of endometriosis (Harada et al., 2001; Van Langendonckt et al., 2002; Defrere et al., 2006); (ii) local immunity has been found to be altered in peritoneal endometriosis (Sidell et al., 2002); (iii) ectopic endometrial cells show cell proliferation and resistance to apoptosis (Nisolle et al., 2000; Beliardi et al., 2004) and (iv) endometriosis is known to be an estrogen-dependent disease and progesterone resistance has also been postulated as a mechanism of progression of endometriosis (Bulun et al., 2006).

Nuclear factor-kappa B (NF-κB) is a transcription factor involved in numerous pathologies and known to be a proinflammatory, mitogenic and antiapoptotic factor in many cell types (Viatour et al., 2005). 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 (Perkins, 2007). Estrogen and progesterone receptors may interact with the NF-κB pathway in a repressive way, but this cross-talk depends on cell type (McKay and Cidlowski, 2005).

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, the most extensively studied dimer being p50/p65 (Karir, 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. 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 and activate the transcription of several target genes (Karir et al., 2004; Hoffmann and Baltimore, 2006).
Different NF-κB activation pathways have been described. The classic or canonical pathway is induced by TNFα and IL-1, among other stimuli, and is dependent on IKKβ activation, which phosphorylates IkBα and mainly results in the activation of p50/p65 heterodimers. The alternative or non-canonical pathway is induced by other stimuli and is dependent on IKKα activation, resulting in the activation of p52/RelB heterodimers. 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-κB subunits with distinct functions (Perkins, 2007).

Human endometrial cells have been shown to express NF-κB proteins (Laird et al., 2000; Page et al., 2002) and to activate NF-κB in response to IL-1β and TNFα in vitro (Sugino et al., 2002; Cao et al., 2005). During menstruation, NF-κB is activated in the glandular epithelium and endothelium of the endometrium (King et al., 2001). In human endometriotic stromal cell cultures, TNFα stimulates activation of NF-κB, increasing IL-8 production, whereas in gonadotropin-releasing hormone (GnRH)-treated patients, there is no such activation (Sakamoto et al., 2003). NF-κB in endometriotic stromal cell cultures activates RANTES, a chemotaxis-related gene, which may play an important role in the pathogenesis of endometriosis (Lebovic et al., 2001).

The binding of p50/p65 dimer to DNA triggers the transcription of genes encoding chemokines, cytokines and adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), which are important for migration of inflammatory and phagocytic cells to tissues where NF-κB has been activated in response to infection or injury (Bonizzi and Karin, 2004). ICAM-1 mRNA and protein have been found to be increased in endometriotic stromal cells, suggesting a role for this adhesion molecule in endometriosis pathophysiology (Viganò et al., 1998).

To date, only a few in vitro studies have suggested that NF-κB may be involved in the transduction of inflammatory signals in endometriosis (Lebovic et al., 2001; Sakamoto et al., 2003; Yamauchi et al., 2004). However, there are no in vivo studies showing NF-κB activation in endometriotic lesions.

The purpose of this study was to investigate the activation status of the NF-κB pathway and expression of the inflammatory marker ICAM-1 in peritoneal endometriotic lesions. The degree of NF-κB activation and IkBα and ICAM-1 expression were studied in red and black endometriotic lesions. Furthermore, the p65 and p50 subunits of active NF-κB dimers were evaluated in endometriotic lesions to gain some insight into the pathways implicated in NF-κB activation.

**Materials and Methods**

**Collection of biopsies**

The use of human tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain. Fifty-four peritoneal endometriotic lesions were obtained during the laparoscopic surgery from women with endometriosis. Lesions were excised by an expert gynecologist with cold scissors, avoiding contamination by peritoneal tissue. Biopsies were transported on ice and immediately stored at −80°C until use.

After protein extraction, protein concentrations were sufficient for analyses in 36 endometriotic lesions (17 red and 19 black lesions). These lesions were obtained from 29 women with a mean age of 29.8 years (range 15–39). Fourteen endometriotic lesions (five red and nine black) were from women taking oral contraception. Lesions were classified according to the revised American Society for Reproductive Medicine (ASRM) classification of endometriosis (Anonymous, 1997). Fourteen lesions were from women with stages I–II endometriosis (4 red and 10 black lesions) and 22 lesions from women with stages III–IV disease (13 red and 9 black lesions).

**Extraction of cytoplasmic and nuclear proteins**

Peritoneal endometriotic lesions were dissected from peritoneal tissue placed on ice, under a magnifying glass, in sterile conditions and then cut into small pieces (~1 mm³) and lysed in a Dounce homogenizer (Wheaton, Millville, NJ, USA). Proteins from the lesion homogenate were extracted using NE-PER® nuclear and cytoplasmic extraction reagents (Perceo Bio Science, Erembodegem, Belgium) according to the manufacturer’s protocol. Protease inhibitors were added to the cytoplasmic and nuclear extraction reagents at the following final concentrations: 0.5 mg/ml benzamidine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.75 mM phenylmethylsulfonyl fluoride to cytoplasmic extracts and 2 mM to nuclear extracts. Phosphatase inhibitors were also added: 1 or 0.5 mM sodium orthovanadate and 2 or 1 mM sodium fluoride to cytoplasmic and nuclear extracts, respectively. Cytoplasmic and nuclear extracts were stored at −80°C until use.

Protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Sample freezing did not alter protein concentrations or quality, as established in preliminary experiments.

**NF-κB activation assays**

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assays (EMSA) were performed on nuclear extracts from endometriotic lesions to measure the level of NF-κB activation. TPA (phorbol 12-myristate, 13 acetate) + CI (calcium ionophore)-stimulated Jurkat nuclear extract (Active Motif Europe, Rixensart, Belgium) was used as a positive control.

DNA binding of NF-κB was assessed using the consensus oligonucleotide of NF-κB (5′-AGT TGA GGC GAC TTT CCC AGG C-3′) (Promega Corp., Madison, WI, USA). The oligonucleotide was radiolabeled with [γ-32P] ATP (GE Healthcare UK Ltd, Amersham Place, Bucks, England), using T4 polynucleotide kinase (New England Biolabs Inc., Ipswich, MA, USA). The labeled probe was purified using the QIAquick nucleotide removal kit (Qiagen Benelux B.V., Venlo, The Netherlands).

For the binding reactions, 10 μg of nuclear proteins were incubated in binding buffer [50 mM Tris·HCl (pH 7.5), 250 mM NaCl, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT and 20% glycerol] with 0.25 μg/ml poly(dI-dC) and 20 μM DNA-protein complexes were then loaded onto a 4% non-denaturing polyacrylamide gel. The gel was run at a constant voltage of 150 V for 2 h in 1× Tris-borate-EDTA buffer. Dried gels were exposed overnight at −80°C to X-Omat autoradiograph film (Eastman Kodak Company, Rochester, NY, USA) for detection.

NF-κB nuclear protein-oligonucleotide complexes were quantified by optical densityometry (OD) of the shifted bands relative to positive controls. The OD of the bands was measured using the Gel Doc 2000 gel documentation system (Bio-Rad Laboratories) and Quantity One 4.0.3 software (Bio-Rad Laboratories).

To check the specificity of the binding reactions, an excess of unlabeled oligonucleotide was added to the reaction 30 min before adding the radiolabeled probe.

For supershift assays, after the first 30 min of incubation in binding buffer, 2 μg of rabbit polyclonal antibody against the p50 subunit and goat polyclonal antibody against the p65 subunit of NF-κB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were added for a further 30 min of incubation at RT before performing the binding reactions.

**NF-κB (p65) DNA-binding activity immunodetection assay**

Activation of NF-κB p65-containing dimers was determined using the TransAM® kit (Active Motif Europe) according to the manufacturer’s instructions.

In brief, DNA binding of p65-containing NF-κB dimers was examined using a 96-well plate with an immobilized oligonucleotide containing an NF-κB consensus-binding site (5′-GGGACCTTCC-3′). Five microgram of total nuclear proteins were incubated in the wells for 1 h at RT. The wells were then washed three times, and 100 μl of p65 subunit mononuclear antibody (1:1000 dilution) was added to each well for 1 h at RT. The wells were then washed three times, and 100 μl of horse-radish peroxidase

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(HRP)-conjugated secondary antibody (1:1000 dilution) was added to each well for 1 h at RT. The wells were then washed four times, and 100 µl of developing solution was added to each well for 5 min. The absorbance was determined on a spectrophotometer (Microplate reader model 550, Bio-Rad) at 450 nm with a reference wavelength of 655 nm. TPA + CI-stimulated Jurkat nuclear extracts were used as positive controls, and 200-fold excess wild-type and mutated NF-κB consensus oligonucleotides (all provided with the kit) were used to monitor the specificity of the assay. To quantify the concentration of activated p65-containing NF-κB dimers in the samples, a standard curve was prepared using recombinant NF-κB (p65) protein (Active Motif Europe).

**Western blotting**

Expression of IkBα and the integral membrane protein ICAM-1 was evaluated by western blot analyses in peritoneal endometriotic lesions. Protein extracts (30 µg per lane) were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. Proteins were then transferred to nitrocellulose membranes (0.45 µm, Bio-Rad) in the Trans Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad) for 30 min at 15 V. The membranes were blocked with 3% blocking grade blocker non-fat milk (Bio-Rad) for 1 h, and then incubated for 1 h at RT with IkBα or ICAM-1 rabbit polyclonal primary antibodies (Santa Cruz Biotechnology, Inc.), which were used at 1:1000 and 1:500 dilutions, respectively. HRP-conjugated goat anti-rabbit secondary antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) was then used at a 1:5000 dilution and incubated for 1 h at RT. Proteins were visualized using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer LAS, Inc., Boston, MA, USA).

Membranes were stripped with Restore™ western blot stripping buffer (Pierce, Rockford, IL, USA) and rebotted with anti-β-actin mouse monoclonal antibody (clone AC-15; Sigma-Aldrich, Saint Louis, Missouri, USA) at a 1:1000 dilution as an internal control for protein loading and transfer. HRP-conjugated goat anti-mouse secondary antibody (Jackson Immuno Research Laboratories, Inc.) was used at a 1:10 000 dilution and incubated for 2 h at RT.

The specificity of the immunoblottings was checked by the use of purified IkBα protein (Santa Cruz Biotechnology, Inc.) as a positive control for IkBα blots, HeLa whole cell lysate (Santa Cruz Biotechnology, Inc.) as a positive control for ICAM-1 blots, and negative controls in the absence of the primary antibody. The OD of the IkBα and ICAM-1 bands relative to the β-actin bands was measured and analyzed using Gel Doc 2000 and Quantity One 4.0.3 software (Bio-Rad Laboratories).

Preliminary experiments showed excellent correlation between the OD of the immunodetected IkBα, ICAM-1 and β-actin bands and the amount of cytoplasmic proteins from endometrial samples loaded into SDS–PAGE (r = 0.96 for IkBα and ICAM-1 and r = 0.98 for β-actin immunoblottings; data not shown).

**Statistical analyses**

General linear models (two-way analysis of variance) determined if data differed between red and black endometriotic lesions, lesions from women using or not using oral contraception, and between stages I–II and III–IV endometriotic lesions. The significance of the interaction between these variables (lesion type, presence or absence of oral contraception and endometriosis classification) was determined using the same model. When an outcome did not show normal distribution, general linear model analysis was conducted on log-transformed data. Data are expressed as means ± SEM. A P-value of 0.05 or less was considered statistically significant. All statistical analyses were performed using NCSS 2004 and PASS trial software (Utah, USA). All the assays were done in duplicate and no differences were noted between repeated experiments.

**Results**

**Constitutive NF-κB activation in peritoneal endometriotic lesions**

Seventeen red and 19 black endometriotic lesions were analyzed by EMSA and constitutive NF-κB DNA-binding activity was observed in all but one lesion. EMSA results on red and black endometriotic lesions are shown in Fig. 1. Analysis of the OD of NF-κB-shifted bands showed a significant 4.1-fold increase (P < 0.001) in the DNA-binding activity of NF-κB dimers in nuclear extracts from red lesions compared with those from black lesions (Fig. 1A and B). Red lesions showed higher NF-κB activation than black lesions obtained from the same patients (n = 3 patients, four red and four black lesions; Fig. 2A). Six red endometriotic lesions were analyzed by supershift assays, and p65- and p50-containing NF-κB dimers were identified in five of them (Fig. 2B). One red lesion showed only p50-containing dimers (data not shown).

EMSA was validated as a quantitative method by preparing a dilution curve with nuclear extracts from one endometrial sample. There was excellent correlation (r = 0.98) between the amount of proteins added to the binding reaction and the OD of the shifted bands (Fig. 2C and D).

To confirm and quantify the constitutive DNA-binding activity of p65-containing NF-κB dimers in endometriotic lesions, 34 nuclear extracts from 16 red and 18 black endometriotic lesions were analyzed by the NF-κB DNA-binding activity immunodetection assay Trans-AM®, which demonstrated the presence of constitutively activated p65-containing NF-κB dimers in endometriotic lesions. Activated NF-κB (p65) mean concentration in nuclear extracts from red lesions (0.18 ± 0.03 ng/µl) was significantly increased (2-fold; P < 0.05) compared with black lesions (0.09 ± 0.02 ng/µl; Fig. 3). Concentrations of DNA-binding p65-containing NF-κB dimers were determined in nuclear extracts of endometriotic lesions by extrapolation from a standard curve prepared using recombinant NF-κB (p65) protein (r = 0.99; data not shown). The specificity of the assay was confirmed using wild-type and mutated NF-κB consensus oligonucleotides, which showed a 5.1-fold increase in activated NF-κB (p65) concentration when mutated oligonucleotide was added to the reaction, as opposed to wild-type oligonucleotide, which inhibited NF-κB-DNA binding (Fig. 3).

EMSA and Trans AM® analyses of endometriotic lesions from women taking or not taking oral contraception did not show any significant differences in NF-κB activation and no significant difference was observed in NF-κB activation between stages I–II and III–IV lesions. The interaction between lesion type (red or black) and oral contraception, as well as lesion type and endometriosis classification, was not significant for EMSA and Trans AM® analyses, indicating that any significant results on NF-κB activation according to the lesion type were independent of the presence or absence of oral contraception and endometriosis stage (data not shown).

**IkBα expression in peritoneal endometriotic lesions**

Cytoplasmic extracts of endometriotic lesions were analyzed by western blotting to quantify the expression of the NF-κB inhibitor, IkBα. Seventeen red and 19 black lesions were examined by western blot analysis. IkBα was strongly expressed in all lesions and no differences were observed between red and black lesions (Fig. 1C and D). No differences were found in the IkBα expression of endometriotic lesions with or without oral contraception, nor was there any significant difference between stages I–II and III–IV lesions.

**ICAM-1 expression in peritoneal endometriotic lesions**

Expression of the integral membrane protein ICAM-1 was evaluated in 17 red and 19 black peritoneal endometriotic lesions by western blotting (Fig. 1). All endometriotic lesions expressed ICAM-1. ICAM-1 expression was significantly higher (1.9-fold) in red endometriotic lesions than black lesions (P < 0.01; Fig. 1E and F). No
significant difference was observed in ICAM-1 expression in lesions from women using or not using oral contraception. ICAM-1 expression analysis of stages I–II and III–IV lesions did not show any significant difference either. The interaction between lesion type and oral contraception or endometriosis classification was not significant for ICAM-1 expression analysis, indicating that the significant result on ICAM-1 expression according to lesion type was independent of the presence or absence of oral contraception and endometriosis stage (data not shown).

**Figure 1.** NF-κB EMSA and western blot analyses of IκBα and ICAM-1 proteins in peritoneal endometriotic lesions. (A) Typical NF-κB EMSA of four nuclear extracts from red lesions (RL) and four nuclear extracts from black lesions (BL); J, stimulated Jurkat cell nuclear extract (positive control). The OD of the shifted bands was measured relative to the OD of the positive control. The specificity of the EMSA was tested by adding an excess of unlabeled oligonucleotide, before adding the radiolabeled probe to the binding reaction (CP, cold probe). (B) Means ± SEM of log-transformed data of OD of shifted bands from 17 red and 19 black endometriotic lesions relative to the OD of the positive control. Red lesions showed significantly greater NF-κB-DNA binding than black lesions ($P < 0.001$). (C) Western blot analyses of IκBα and β-actin of four red and four black endometriotic lesions and the positive control (purified IκBα). (D) Means ± SEM of OD of IκBα bands relative to β-actin bands (internal control) in 17 red and 19 black endometriotic lesions. No differences were observed in IκBα expression between red and black endometriotic lesions. (E) ICAM-1 and β-actin western blots of four red and four black endometriotic lesions and the positive control: H (HeLa whole cell lysate). (F) Means ± SEM of OD of ICAM-1 bands relative to β-actin bands in 17 red and 19 black endometriotic lesions. Red lesions showed significantly higher expression of ICAM-1 than black lesions ($P < 0.01$).

**Discussion**

Activation of the transcription factor NF-κB is involved in many inflammatory diseases and cancer (Viatour et al., 2005). NF-κB activity is constitutive in B cells and in some monocyte cell lines, but in most other cells, it is very low or undetectable (Rice and Ernst, 1993). NF-κB transcriptional activity regulates many genes implicated in inflammation (IL-1, IL-6, IL-8, iNOS and COX-2), immune regulation (IFN-γ, TNF-α, RANTES and ICAM-1),
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apoptosis and cell proliferation (Bax, Fas, Fas-L, cyclin D1, c-Myc and EGF), angiogenesis (VEGF) and NF-κB feedback regulation (IxBo, p100 and p105) (Aggarwal, 2004; Karin, 2006). Many of these NF-κB-regulated genes have been implicated in the physiopathology of endometriosis (Lebovic et al., 2001; Sakamoto et al., 2003; Iba et al., 2004; Yamauchi et al., 2004; Horie et al., 2005).

NF-κB-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). In vitro studies show that NF-κB can be activated in endometriotic stromal cells by known inducers of the classic NF-κB pathway such as IL-1β, TNF-α or lipopolysaccharide (Lebovic et al., 2001; Sakamoto et al., 2003; Iba et al., 2004), and activation of the NF-κB pathway has been demonstrated in non-stimulated endometrial epithelial cells, endometrial stromal cells and endometriotic stromal cell cultures (Tamura et al., 2002; Han and Sidell, 2003; Horie et al., 2005). To date, however, there has been no direct evidence of constitutive NF-κB DNA-binding activity in endometriosis in vivo.

In this study, using two different and complementary methods (EMSA-supershift and TransAM®), we were able to demonstrate, for the first time, that NF-κB is constitutively activated in peritoneal endometriotic lesions and that red endometriotic lesions show a significantly higher degree of activation of the NF-κB pathway than black lesions. EMSA and supershift assays revealed DNA-binding activity of p50/p65 and p50-containing NF-κB dimers, whereas TransAM® allowed quantification of p65-containing dimers. The concentration of active p65-containing dimers was significantly higher in red endometriotic lesions than black lesions, which is important, since p50/p65 are transcriptionally active dimers, whereas the p50/p50 homodimer functions as a repressor, since it lacks a transcription activation domain (Bonizzi and Karin, 2004).

Another finding of this study was that ICAM-1 expression, like NF-κB activation, is significantly increased in red endometriotic lesions compared with black lesions. Since the promoter region of ICAM-1 gene contains putative recognition sequences for NF-κB (Chen et al., 2000), it is highly plausible that increased ICAM-1 expression results from the activation of NF-κB in endometriotic lesions.

Lesion type was the only factor influencing NF-κB activation and ICAM-1 expression in endometriotic lesions, since no statistically significant differences were found between lesions from women taking or not taking oral contraception or stages I–II and III–IV lesions.

The different levels of NF-κB activation and ICAM-1 expression observed in red and black endometriotic lesions strongly suggest a more extensive inflammatory response in red endometriotic lesions than black lesions. Red lesions 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). Our data are consistent with other studies that have shown differential...
inflammatory reactions, vascularization, and mitotic and apoptotic activity in red and black endometriotic lesions.

The more intense inflammatory reaction observed in red endometriotic implants is substantiated by other studies showing that macrophage infiltration, matrix metalloproteinase-1 (MMP-1) mRNA expression and macrophage migration inhibitory factor (MIF) are increased in red endometriotic lesions compared with black lesions (Kokorine et al., 1997; Kats et al., 2002; Khan et al., 2004). MMP-1 and MIF expression have been shown to be up-regulated by NF-κB in macrophages and endometrial stromal cells (Bondeson et al., 1999; Cao et al., 2006). A recent study by Lawson et al. (2007) showed higher expression of IL-1 receptor type 1 (IL-1RI) in red endometriotic lesions than black and white lesions, which corroborates our findings, since this receptor is one of the main receptors activating the NF-κB pathway. Increased expression of IL-1RI could be one of the factors responsible for the increased constitutive NF-κB activation and inflammatory response in red endometriotic lesions.

The observation of a more important vascularization pattern in red endometriotic lesions is supported by the fact that vascular endothelial growth factor (VEGF) and microvessel density have been found to be higher in red endometriotic lesions than in black lesions (Donnez et al., 1998; Khan et al., 2003). VEGF has been shown to be positively modulated by NF-κB in macrophages (Kiriaikidis 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 in black lesions (Donnez et al., 1998; Khan et al., 2003). HGF has been shown to activate the NF-κB pathway in rat hepatocytes, and survivin to be up-regulated by NF-κB activation in human multiple myeloma cells (Mitsiades et al., 2002; Kaibori et al., 2006).

All these findings, as well as our results, confer to red endometriotic lesions a major status of inflammation, vascularization, proliferation and resistance to apoptosis. The NF-κB pathway therefore looks to be a promising therapeutic target for endometriosis, since it plays a central role in these biological processes.

Regarding the supershift assay analysis, our results suggest that the classic pathway could be the main activation pathway of NF-κB in endometriotic lesions. The fact that only p50-containing NF-κB dimers were found in supershifted nuclear extracts strongly suggests that the alternative or non-canonical NF-κB pathway is not functional in endometriotic lesions, since it activates mainly p52/RelB NF-κB dimers, and is associated with the development of lymphoid organs (Hoffmann and Baltimore, 2006). In contrast, the classic or canonical NF-κB pathway is known to activate p50/p65 NF-κB dimers in response to inflammatory stimuli such as IL-1β and TNF-α, which have been shown to activate NF-κB in endometrial and endometriotic stromal cells (Lebovic et al., 2001; Sugino et al., 2002; Sakamoto et al., 2003; Karin et al., 2004; Cao et al., 2005). Moreover, the increased expression of IL-1RI observed in red endometriotic lesions (Lawson et al., 2007) provides further evidence that the classic pathway of NF-κB activation is the main pathway activating NF-κB in endometriotic lesions. Since activation of the classic pathway is dependent on IKKβ activation, this kinase warrants investigation as a potential target for new endometriosis therapies. However, activation of atypical NF-κB 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 (Perkins, 2007), but they have not been studied in the context of endometriosis.

The NF-κB inhibitor, IκBα, was found to be equally expressed in red and black peritoneal endometriotic lesions. NF-κB regulation involves negative feedback inhibition through NF-κB-directed synthesis of IκBα (Chiao et al., 1994). IκBα enters the nucleus, removes NF-κB from the DNA and then exports it to the cytoplasm, restoring the pool of inactive NF-κB/IκBα 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 IκBα proteins (Rice and Ernst, 1993), which could also occur in endometriotic cells.

In conclusion, this is the first study to demonstrate constitutive NF-κB activation in peritoneal endometriotic lesions collected from women. Differential levels of NF-κB DNA-binding activity and ICAM-1 expression 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. In addition, this study offers further insight into the pathways implicated in NF-κB activation in endometriotic lesions, showing the involvement of p50/p65 dimers. Constitutive activation of the NF-κB pathway could explain the inflammatory response, cell proliferation and resistance to apoptosis observed in endometriotic lesions, making the NF-κB pathway an attractive therapeutic target for the treatment of endometriosis.

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