REPRODUCTION

Nuclear factor κB pathway and interleukin-6 are affected in eutopic endometrium of women with endometriosis

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Abstract

In order to investigate the role of the nuclear factor κB (NFKB) pathway on gene expression in the eutopic endometrium in endometriosis, and in particular of interleukin-6 (IL6), we evaluated RELA, IκB kinase (CHUK), NFKBIA and IL6 expressions and NFKB DNA binding in eutopic endometrium from women with endometriosis. Eutopic endometrium was obtained from 37 women with endometriosis and 42 fertile women during laparoscopy. We analysed *RELA*, *CHUK*, *NFKBIA* and *IL6* mRNA levels (RT-PCR); RELA, CHUK and NFKBIA proteins and p-NFKBIA/NFKBIA ratio (western blot); and NFKB binding (DNA shift assay) and IL6 concentration (ELISA) in endometrial explants. Our results indicate that mRNA and cytoplasmic proteins of RELA and CHUK exhibit constant levels in normal endometrium during the menstrual cycle. A dramatic increase ($P < 0.05$) in *NFKBIA* mRNA expression, RELA nuclear presence and the mRNA and the protein of IL6 during late secretory phase was also observed in this tissue. By contrast, in eutopic endometrium from endometriosis patients, a decrease ($P < 0.05$) in *IL6* mRNA and protein (61%), *NFKBIA* mRNA (46%), p-NFKBIA/NFKBIA ratio (42%), RELA nuclear stromal (68%) and CHUK (48%) proteins were found exclusively during the late secretory phase compared with normal endometrium. In conclusion, the canonical activation of NFKB pathway is deregulated and may have reduced transcriptional function affecting NFKBIA and IL6 expression, genes related local proinflammatory processes. These molecular alterations observed during the late secretory phase in eutopic endometrium from endometriosis patients constitute a NFKB system dysfunction, suggesting that NFKB could be an important factor in endometriosis aetiology.

Introduction

Endometriosis, a common gynaecological disorder affecting up to 10% of the reproductive-aged female population, is characterized by the presence and growth of endometrial tissue outside the uterine cavity and is associated with chronic pelvic pain and reduction of fertility (Barlow & Kennedy 2005, Anaf *et al.* 2006). Although its aetiology and pathogenesis remain elusive, the most accepted theory proposes that uterine endometrial fragments contained in menstrual flux and regurgitated into the peritoneal cavity could exhibit ectopic colonizing ability (Sampson 1940). Immune and inflammatory alterations at the cellular and molecular levels in endometriosis could contribute to the endometriotic implant survival and growth, and thus may also affect fertility and cause chronic pain with the onset of a local inflammatory reaction.

Cytokines are regulatory peptides produced by several nucleated cell types in the body and have pleiotropic regulatory effects on many cell types. Leukocytes, including macrophages, are important cytokine producers (Kayisli *et al.* 2002, Berkkanoglu & Arici 2003). In the endometrium, cytokines are implicated in conferring embryo receptivity and preparing it for menstrual shedding (Tabibzadeh *et al.* 1995, 1999, Sherwin *et al.* 2004). Deregulated expression of proinflammatory cytokines in the endometrium has been associated with female fertility reduction and abortion recurrence (Von Wolff *et al.* 2000, Kao *et al.* 2003). Therefore, cytokines may participate in the pathophysiology of endometriosis contributing to the establishment and proliferation of ectopic endometrial implants. Several authors have reported augmented cytokine concentration in peritoneal fluid in endometriosis secreted by macrophages, thus indicating adverse changes in the hormonal, chemical and/or cellular milieu (Bedaiwy *et al.* 2002, Cheong *et al.* 2002).

The cytokine interleukin-6 (IL6) has been associated with several different functions, including embryo implantation and menses (Von Wolff *et al.* 2000). In the endometrium, its expression is mainly observed in epithelial cells; its mRNA levels are reduced in the

proliferative and early secretory phase while they are increased in mid and late secretory phases, thus indicating a negative regulation by oestradiol (Von Wolff *et al.* 2000). This cytokine is under the control of nuclear factor κB (NFKB), the central regulator of the immune system gene expression, which also plays a regulatory role in reproductive tissues (Laird *et al.* 2000, King *et al.* 2001).

NFKB is an important transcription factor mainly involved in inflammatory and immune responses. Its function was recognized in tumour necrosis factor (TNF) and IL1B receptors signal transduction pathways in several cell types where it activates multiple genes, including *IL6* (Laird *et al.* 2000, Sakamoto *et al.* 2003). The NFKB family is basically integrated by RELA, NFKB2, NFKB1 (p50) and REL subunits, proteins that contain a DNA-binding domain, a dimerization (or RHD) domain and a nuclear localization signal domain. These proteins may form homo- or heterodimers, but the more common complex found *in vivo* is RELA–NFKB1; RELA is the subunit that most frequently participates in the active NFKB complex (Beinke & Ley 2004, Chen & Green 2004). Commonly, NFKB is located in the cell cytoplasm bound to its inhibitory protein IκB through its RHD domain, which blocks the nuclear location signal and maintains the transcription factor sequestered in the cytoplasm. In response to an extracellular signal, such as TNF or IL1A, the signalling cascade is activated and IκB is phosphorylated by IκB kinase (IKK), allowing the release of NFKB and the exposition of its nuclear location signal. Then, NFKB translocates into the nucleus, binds to NFKB DNA elements and induces its target genes, such as *IL6*, and its own inhibitory protein, NFKBIA (Pahl 1999, Renard & Raes 1999, Chen & Green 2004; Fig. 1).

It is well known that ovarian steroids are the main regulators of endometrial function during the menstrual cycle. Probably, they may also exert a regulatory effect on the NFKB system through their specific receptors, via direct or indirect interaction with the RELA subunit, forming an inactive heterodimeric complex that may affect the expression of certain genes as it was described in other tissues that co-express both types of molecules (Kalkhoven *et al.* 1996, Han & Sidell 2003, Ghisletti *et al.* 2005). In endometriosis, the different isoforms of oestradiol and progesterone receptors have an abnormal expression in ectopic endometrium (Attia *et al.* 2000, Matsuzaki *et al.* 2000, Hudelist *et al.* 2005) that may be affecting NFKB function and, therefore, local cytokine expression.

All the components of the NFKB system have been described in normal endometrium (Laird *et al.* 2000, King *et al.* 2001), and there are some reports of the role of the NFKB system in benign endometrial hyperplasia and endometrial cancer (Vaskivuo *et al.* 2002), both pathologies that present deregulated tissue homeostasis. However, the expression of these proteins in the eutopic endometrium in endometriosis, a disease that also
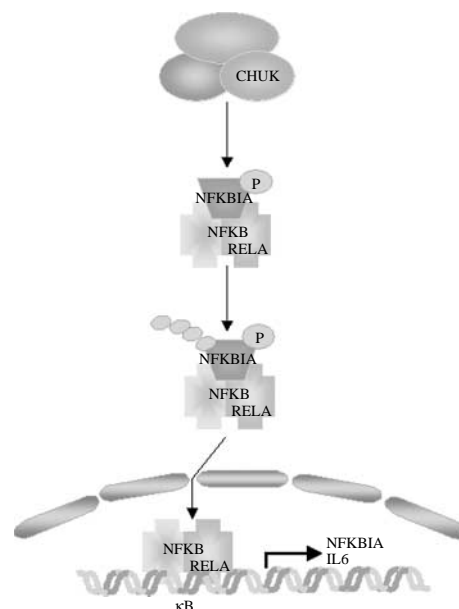

Figure 1 Molecules of NFKB signalling pathway studied. Activation of IKK complex results in IκB phosphorylation and degradation. Then, NFKB translocates into the nucleus, binds to NFKB1 DNA elements and induces transcription of target genes such as *IL6* and *NFKBIA*.

presents abnormal endometrial homeostasis with reduced apoptosis (Meresman *et al.* 2000, Braun *et al.* 2002, Johnson *et al.* 2005), is not clear.

In order to investigate the role of the NFKB system on gene expression in the eutopic endometrium in endometriosis, and in particular of IL6, we evaluated the expression of RELA subunit, the activator CHUK, the inhibitor NFKBIA, NFKB DNA binding and the cytokine IL6 in eutopic endometrial tissue from women with endometriosis and compared them with endometrium from control women. The molecules studied in the present work are shown in Fig. 1.

Results

RELA, CHUK and NFKBIA mRNA

By RT-PCR, the mRNA of *RELA*, *CHUK* and *NFKBIA* were detected in eutopic endometrium from women with and without endometriosis. The mRNA of *RELA* and *CHUK* showed a constant pattern throughout the menstrual cycle in both types of endometria (Table 1). In normal endometrium *NFKBIA* mRNA levels were also constant from the proliferative to the mid-secretory phases, but at the late secretory phase a 30% increase ($P < 0.05$ compared with proliferative phase) was observed. By contrast, in eutopic endometrium from women with endometriosis, *NFKBIA* mRNA levels were constant during all of the menstrual cycle, being reduced 46% at the late secretory phase compared with normal endometrium ($P < 0.05$; Table 1).

Table 1 *RELA, CHUK, NFKBIA* mRNA levels in normal endometrium and in eutopic endometrium of women with endometriosis during the menstrual cycle.

Menstrual cycle stage	RELA		CHUK		NFKBIA	
	Normal	Endometriosis	Normal	Endometriosis	Normal	Endometriosis
Proliferative	0.81 ± 0.04	0.80 ± 0.02	0.41 ± 0.04	0.46 ± 0.06	0.47 ± 0.06	0.50 ± 0.11
Early secretory	0.75 ± 0.06	0.77 ± 0.04	0.43 ± 0.05	0.44 ± 0.04	0.40 ± 0.15	0.33 ± 0.13
Mid-secretory	0.77 ± 0.04	0.81 ± 0.06	0.44 ± 0.04	0.49 ± 0.03	0.33 ± 0.13	0.47 ± 0.10
Late secretory	0.69 ± 0.04	0.80 ± 0.05	0.36 ± 0.03	0.43 ± 0.02	$0.61 \pm 0.06^{\dagger}$	$0.33 \pm 0.06*$

RT-PCR was performed in eutopic endometrium obtained from women with and without endometriosis during menstrual cycle. Results are given as mean±s.e.m. of four normal endometria and four eutopic endometria of women with endometriosis in each stage of the menstrual cycle corresponding to 32 specimens. *$P < 0.05$ compared with normal endometrium. $^{\dagger}P < 0.05$ compared with proliferative phase.

CHUK and NFKBIA proteins

During the menstrual cycle, CHUK and NFKBIA proteins were detected in endometrial cytoplasm obtained from women with and without endometriosis. In normal endometrium, CHUK cytoplasmic protein levels were constant during all of the menstrual cycle; in eutopic endometrium from endometriosis patients, this protein was constant in the majority of the cycle, but a significant reduction (48% compared with normal endometrium, $P < 0.05$) during the late secretory phase was detected (Fig. 2A).

In contrast to the increase in *NFKBIA* mRNA levels at the late secretory phase observed in normal endometrium, the NFKBIA protein was constant in the uterine cell cytoplasm during the menstrual cycle; similar results were obtained in eutopic endometrium from women with endometriosis, where levels of the NFKBIA cytoplasmic protein did not change at any stage of the menstrual cycle (data not shown). The phosphorylated NFKBIA (p-NFKBIA) form was also detected in the cytoplasmic fraction in endometrium from both groups of women with and without endometriosis (Fig. 2B). The ratio of p-NFKBIA/NFKBIA, with a slight predominance of the unphosphorylated form, was constant during the menstrual cycle in normal endometrium. However, in eutopic endometrium from endometriosis patients at the late secretory phase, this ratio decreased 62% compared with proliferative phase ($P < 0.05$) and 42% compared with late secretory normal endometrium ($P < 0.05$; Fig. 2B).

RELA protein and NFKB DNA binding

The dissociation of NFKB from NFKBIA permits NFKB translocation to the nucleus where it binds to specific sequences in the promoter regions of target genes. By immunoblot, the RELA cytoplasmic protein was constant during the menstrual cycle in endometrium from women with and without endometriosis; however, in normal endometrium, RELA nuclear protein increased throughout the menstrual cycle, reaching the highest levels at the late secretory phase (Table 2). Although RELA nuclear protein was higher at proliferative phase and lower at the late secretory phase in endometrium from endometriosis patients these differences were not statistically different from normal endometrium ($P = 0.083$ and 0.093 respectively).

Similar to immunoblot, the nuclear protein homogenate obtained from normal endometrium at the late secretory phase of the menstrual cycle was markedly bound to target NFKB consensus oligonucleotides compared with the early secretory phase ($P < 0.05$), an increase that was not observed in eutopic endometrium from women with endometriosis, which presented a constant binding throughout the menstrual cycle. At the
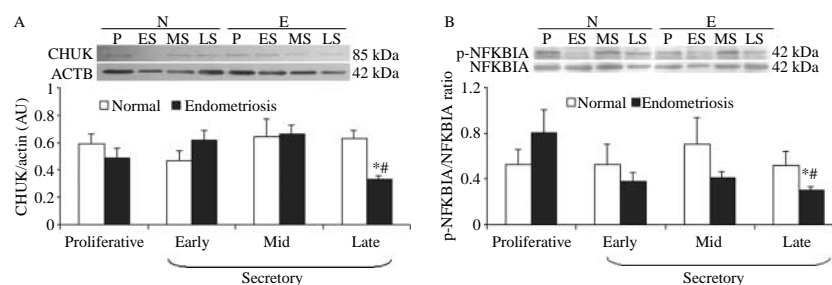

Figure 2 (A) Immunoblot of CHUK and (B) ratio of p-NFKBIA/NFKBIA protein in normal endometrium (N) and eutopic endometrium from endometriosis patients (E). Proteins electrophoretically transferred to membrane were incubated with rabbit anti CHUK, NFKBIA and p-NFKBIA or mouse anti β-actin. Representative gel is shown. Data are the mean±s.e.m. of four normal endometria and four endometria from endometriosis patients in each stage of the menstrual cycle corresponding to 32 tissues. *$P < 0.05$ compared with normal endometrium and $^{\#}P < 0.05$ compared with proliferative phase.

Table 2 Cytoplasmic and nuclear RELA proteins in normal endometrium and in eutopic endometrium of women with endometriosis during the menstrual cycle.

Menstrual cycle stage	Normal		Endometriosis	
	Cytoplasm	Nucleus	Cytoplasm	Nucleus
Proliferative	0.90 ± 0.10	0.44 ± 0.13	1.14 ± 0.06	0.83 ± 0.10
Early secretory	0.66 ± 0.13	0.58 ± 0.20	1.14 ± 0.11	0.68 ± 0.09
Mid-secretory	1.29 ± 0.21	0.66 ± 0.25	1.03 ± 0.14	0.70 ± 0.12
Late secretory	0.92 ± 0.16	$0.92 \pm 0.08^*$	0.69 ± 0.11	0.68 ± 0.07

Immunoblot was performed in eutopic endometrium obtained from women with and without endometriosis during menstrual cycle. Results are given as mean±s.e.m. of four normal endometria and four eutopic endometria of women with endometriosis in each stage of the menstrual cycle corresponding to 32 specimens. $*P < 0.05$ compared with proliferative phase.

late secretory phase, NFKB DNA binding was reduced 70% in the endometrium from the patients compared with normal tissue, although this difference was not statistically significant ($P = 0.09$; Fig. 3).

The immunohistochemistry of RELA exhibited a wide immune-detection in the epithelial cell surface (data not shown), epithelial glands, stromal cells and endothelial cells from normal endometrium and eutopic endometrium from endometriosis patients (Fig. 4A–D). Cytoplasmic and nuclear immunostaining was detected in the endometrium of women with and without endometriosis. The percentage of cytoplasmic RELA-positive cells was not altered during the menstrual cycle and a homogeneous distribution of the brown positive staining was present in the majority of epithelial cells and in some stromal cells from normal women and women with endometriosis. Similar to immunoblot, the presence of RELA was lower in the nucleus than that in the cytoplasm in stromal and epithelial cells from both endometrium types during the menstrual cycle. In stromal cells from normal endometrium, but not in epithelial cells, an increase was observed in nuclear

RELA-positive cells at the late secretory phase (115% compared with proliferative phase; $P < 0.05$), and although the RELA nuclear/cytoplasmic ratio was also increased (72%) at this stage, this increase was not statistically different ($P = 0.092$) from proliferative phase (Fig. 5A and C). By contrast, in the stroma of eutopic endometrium from endometriosis patients, the nuclear presence and the nuclear/cytoplasmic ratio of RELA were reduced 68% and 52% respectively ($P < 0.05$) compared with the normal endometrium at the late secretory phase (Fig. 5A and C); these changes were not observed in the epithelial cell compartment (Fig. 5B and D).

mRNA and concentration of IL6

The expression of *IL6* mRNA increased constantly from early to late secretory phase in normal endometrium. In the eutopic endometrium from endometriosis patients, the *IL6* mRNA level was similar to normal endometrium in the proliferative, early and mid-secretory phases; nevertheless, in the late secretory phase, the *IL6* mRNA level was significantly reduced (61%) when compared with late secretory normal endometrium (Fig. 6A). The concentration of *IL6* was 61% reduced in endometrial homogenates obtained from women with endometriosis compared with normal endometrium from the mid and late secretory phases ($P < 0.05$; Fig. 6B).

Discussion

The NFKB system, constituting many proteins, is an important gene regulator with more than 150 genes related to immune and inflammatory responses, cell proliferation and cell death lying under its control (Pahl 1999, Beinke & Ley 2004). The present study describes and characterizes some components of this system in eutopic endometrium from endometriosis patients and
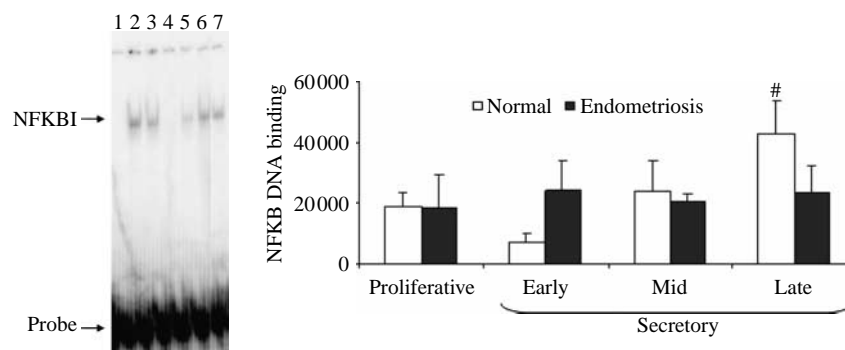

Figure 3 Functional analysis of NFKB DNA consensus sequence binding to endometrial nuclear extract from normal and endometriosis women. Representative DNA shift assay. Nuclear extract from normal endometrium: lane 2 (late secretory phase), lane 4 (mid-secretory phase; incubated with 100-fold of unlabelled NFKB1 oligonucleotide) and lane 7 (mid-secretory phase); nuclear extract of eutopic endometrium from endometriosis patients: lanes 3 and 5 (late secretory phase) and lane 6 (mid-secretory phase); lane 1 (free probe). Data are the mean±s.e.m. of five normal endometria and five endometria from endometriosis patients in each stage of the menstrual cycle corresponding to forty tissues. $^{\#}P < 0.05$ compared with the early secretory phase.
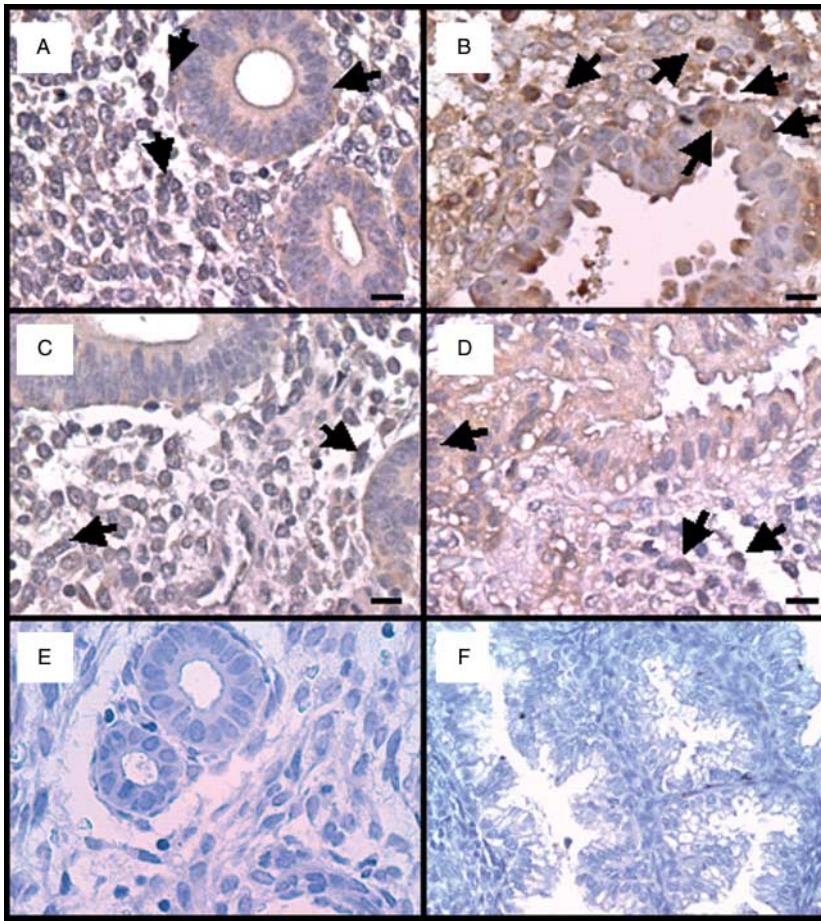
Figure 4 Immunohistochemical localization of RELA in normal endometrium and eutopic endometrium from endometriosis patients. (A and B) Normal endometrium and (C–F) eutopic endometrium from endometriosis patients were stained with RELA using DAB as chromogen as indicated in Materials and Methods. (E and F) Negative control. Proliferative phase: A, C and E; late secretory phase: B, D and F. (Scale bar, 20 µm).

evaluates its regulatory role on IL6, an important proinflammatory cytokine in endometrial function.

Our results indicate that mRNA and cytoplasmic protein of RELA and CHUK exhibit constant levels during the menstrual cycle in normal endometrium, in contrast to *NFKBIA* mRNA expression, RELA nuclear presence and NFKB DNA binding, which present an increase during the late secretory phase, suggesting

negative regulation by progesterone in agreement with King *et al*. (2001). Because *NFKBIA* is one of the genes regulated by NFKB (Gilmore 1999, Pahl 1999), this change related to the menstrual cycle indicates that the NFKB system functions as a gene modulator in human endometrium in accordance with Laird *et al*. (2000). On the contrary, in eutopic endometrium from women with endometriosis, the CHUK protein was strongly reduced
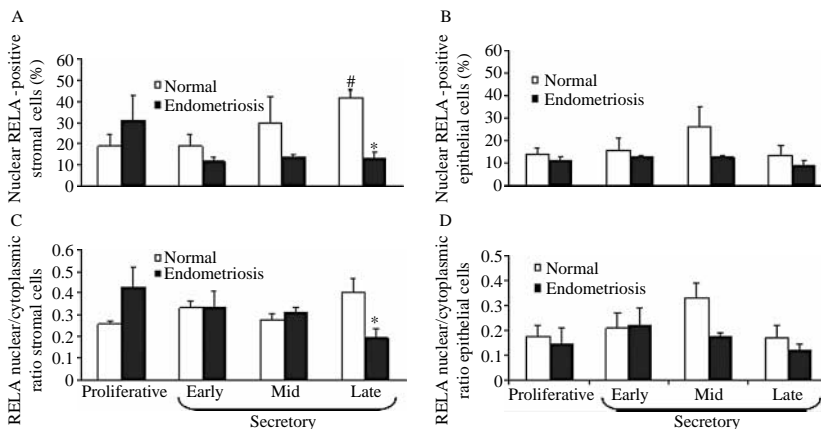

Figure 5 Immunohistochemical detection of nuclear RELA in (A) stromal and (B) epithelial cells, and RELA nuclear/cytoplasmic ratio in (C) stromal and (D) epithelial cells from women with and without endometriosis. Positive nuclear staining was detected in stromal and epithelial cells. The data are the mean ±S.E.M. of four normal endometria and four endometria from endometriosis patients in each stage of the menstrual cycle corresponding to 32 specimens. *P<0.05 compared with normal endometrium and #P<0.05 compared with proliferative phase.
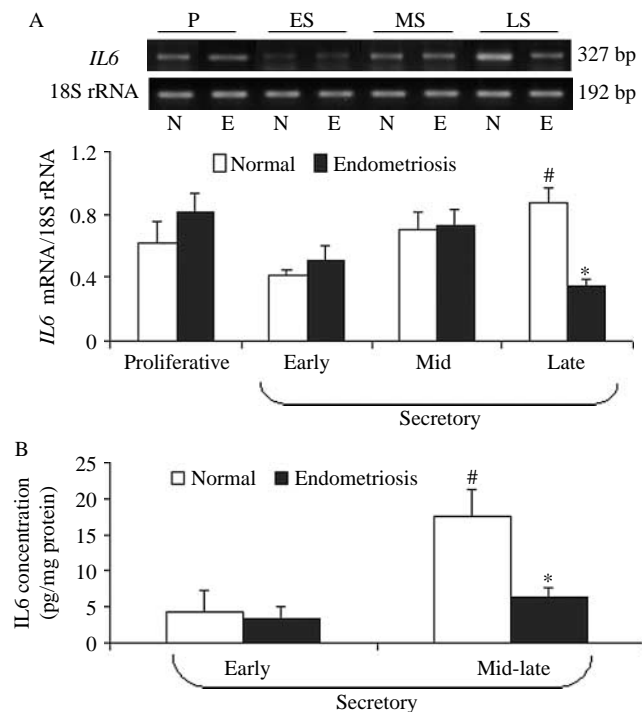
Figure 6 Expression of (A) mRNA and (B) protein of IL6 in normal endometrium and eutopic endometrium from endometriosis patients. Endometrial cDNA prepared from each stage of the menstrual cycle was amplified as described in Materials and Methods; all data were normalized to 18S rRNA levels (A). IL6 protein was detected in secretory endometrium homogenates by ELISA (B). Results are the mean±S.E.M. of 8 and 6 proliferative and 15 and 21 secretory endometria from normal and endometriosis women respectively corresponding to 5 and 7 endometria in each stage of the secretory phase (A), and 3 early secretory and 5 mid and late secretory endometria from eight normal women and eight women with endometriosis. *$P<0.05$ compared with normal endometrium and #$P<0.05$ compared with proliferative phase.

in the late secretory phase, coincidently with the reduction of *NFKBIA* mRNA, p-NFKBIA/NFKBIA protein ratio, RELA nuclear presence, RELA nuclear/cytoplasmic ratio and NFKB DNA binding when compared with the normal endometrium at the same stage of the menstrual cycle. The decrease in CHUK protein by defects in translation or stability may be involved in NFKBIA phosphorylation reduction, thus preventing its degradation and therefore keeping RELA sequestered in the endometrial cell cytoplasm, blocking its nuclear translocation and gene-activating function. This decrease may also be affecting NFKBIA expression, one of the genes regulated by NFKB. These findings are in agreement with the reduced binding of NFKB to consensus oligonucleotide exhibited by eutopic endometrium from these patients and exclusively at the late secretory phase.

The selectively regulated RELA presence only in the nucleus of stromal cells during the menstrual cycle in normal endometrium may explain the lack of significant difference in the reduction of RELA content and NFKB DNA binding in eutopic endometrium from endometriosis patients studied in tissue homogenates that included both endometrial cell compartments. On the other hand, NFKB consists of heterodimers that participate in the NFKB DNA binding, and although RELA is the main subunit acting in reproductive tissues, in the present work this aspect was not evaluated. The fact that phosphorylation of NFKBIA in two serine residues by CHUK is a prerequisite for the activation of gene expression mediated by NFKB (Tanaka *et al.* 2001, Viatour *et al.* 2005) is in accordance with these results. However, we cannot rule out that CHUK cytoplasmic protein decrease at the late secretory phase may be in concordance with an increase in nuclear CHUK because we did not analyse its nuclear presence as mentioned by Park *et al.* (2005) in mammary cell line MCF7. Overall, these results indicate that the NFKB system presents anomalies that negatively affect its functions during the late secretory phase of the menstrual cycle in eutopic endometrium from women with endometriosis.

The alterations found in the present study may be involved in other processes affected in endometriosis, such as cellular survival and the proinflammatory and immune responses, also partially regulated by NFKB. It is known that in this disease several genes are expressed in aberrant forms, affecting cell proliferation and cell death in eutopic endometrium, which might favour the ectopic endometrial cell survival that may be involved in its aetiology (Johnson *et al.* 2005, Zhang *et al.* 2006). Substantial evidence links NFKB to antiapoptotic and proapoptotic effects, through the BCL2 family and the FAS/FASLG complex (Lee *et al.* 1999, Pahl 1999, Wang *et al.* 1999, Harada *et al.* 2004). In ectopic and/or eutopic endometria from endometriosis patients, endo-metrial cell viability is mainly controlled by the over-expression of BCL2 and a decreased expression of BAX and FAS molecules (Meresman *et al.* 2000, Garcia-Velasco *et al.* 2002, Johnson *et al.* 2005), effects that may allow cell survival in ectopic sites. Although the reduced function of the NFKB system observed in our results does not explain the mechanism by which apoptosis is reduced in eutopic endometrium from endometriosis patients, in other pathologies such as adenomyosis, defined as the presence of ectopic endometrium within the myometrium, the decrease in apoptosis was regulated by mediators other than BCL2 (Yang *et al.* 2007), while in breast cancer and in B-cell leukaemia an association between enhanced expression of BCL2 and NFKB2, other NFKB heterodimers, was shown (Viatour *et al.* 2003). Furthermore, the proteins TNFAIP3 and BCL2A1, also regulated by NFKB, can partially protect the cells from apoptosis induced by TNF or FAS (Opipari *et al.* 1992, Lee *et al.* 1999, Wang *et al.* 1999). On the other hand, T cells can be induced to undergo apoptosis upon T-cell receptor stimulation, also through

the NFKB-dependent upregulation of FASLG (Kasibhatla *et al*. 1999). Then, this system regulates cell viability at multiple levels including transcriptional and post-translational activation. However, in late secretory eutopic endometrium from endometriosis patients, both the NFKB system and cell viability need additional study.

The proinflammatory cytokine IL6, the other gene regulated by NFKB evaluated in this study, showed a temporal and spatial expression in normal endometrium in accordance with the previous reports (Vandermolen & Gu 1996, Von Wolff *et al*. 2000). The reduced levels of IL6 observed exclusively at the late secretory phase in eutopic endometrium from endometriosis patients are probably related to the reduction of CHUK protein, *NFKBIA* mRNA and p-NFKBIA/NFKBIA protein ratio, both of which are proteins involved in the mechanism of NFKB activation that may affect RELA nuclear location, RELA nuclear/cytoplasmic ratio and NFKB DNA binding. Interestingly, the cytokine reduction and the maximal leukocyte infiltration coincide in eutopic endometrium from endometriosis patients at the late secretory phase, as was observed using an antibody against the common leukocyte antigen PTPRC (MC Johnson, abstract 286, 10th World Congress on Endometriosis, Melbourne, Australia, 2008), suggesting that the cytokine reduction was an endometrial cell alteration rather than a reduction of infiltrating leukocyte number. Nevertheless, several immunological anomalies such as increased active macrophages with decreased phagocytic ability, decreased number and activity of natural killer cells and augmented CD4:CD8A ratio at peritoneal fluid and also peritoneum and ectopic endometrium have been related to endometriosis pathogenesis by several authors (Lebovic *et al*. 2001, Szyllo *et al*. 2003, Khan *et al*. 2004, Wu *et al*. 2005).

These results give evidence of the transcriptional dysfunction of the NFKB during the late secretory phase in the eutopic endometrium from women with endometriosis, similar to that observed in recurrent abortion cases in which deregulation of IL6, IL1B, IL11 and LIF, cytokines related to or dependent on this system, were observed (Von Wolff *et al*. 2000, Dimitriadis *et al*. 2006, Mikolajczyk *et al*. 2006). IL6 participates in endometrial function by acting as a mediator in the maternal-embryo peri-implantational communication, endometrial immune-tolerance and on inflammatory events in the implantation site (Sengupta *et al*. 2003). Although we found a reduction in IL6 only at the late secretory phase, the contribution of this cytokine on subfertility associated to endometriosis may not be ruled out.

In addition, IL6 has been associated with cellular viability and cellular differentiation. Its participation in mammary gland involution by mediating the expression of BAX, probably through the MAPK pathway (Zhao *et al*. 2002), and its poor expression in some human breast cancer has been reported (Hu *et al*. 2004). This evidence suggests that IL6 may play a regulatory role on endometrial homeostasis through proapoptotic mechanisms in the peri-menses stage coincident with their maximal expression. This function in endometrial cell viability may be negatively affected in endometriosis, as indicated by the local reduction of IL6 levels at the late secretory phase concomitant with the decrease in the apoptosis rate during the same phase (Johnson *et al*. 2005, Zhang *et al*. 2006).

The results presented in this work do not agree with the previous reports that indicate a constitutive NFKB activation in peritoneal endometriotic lesions (Gonzalez-Ramos *et al*. 2007, Nasu *et al*. 2007), a high serum IL6 concentration that provides a promising serum non-invasive marker of endometriosis (Martinez *et al*. 2007, Othman *et al*. 2008) and a differential response of IL6 secretion in control, eutopic and ectopic endometrial stromal cell cultures from normal and endometriosis women (Tseng *et al*. 1996). This is a proinflammatory disease, with an increased peritoneal fluid volume that contains increased concentrations of white blood cells and activated peritoneal macrophages, which together with endometriotic lesions and macroscopically normal peritoneum are significant contributors to IL6 production and also other inflammatory and invasive molecules mediated by local NFKB activation (Hastings & Fazleabas 2006, Kyama *et al*. 2006, Ulukus *et al*. 2006, Lousse *et al*. 2008, Montagna *et al*. 2008). In the present study, IL6 was exclusively analysed in *ex vivo* eutopic endometrium, making a difference from those reports using ectopic endometrium or stromal cell cultures in which the paracrine communication of cell compartment was lost. In addition to and supporting our point, recently Salsami *et al*. (2008) and previously Bergqvist *et al*. (2001) have reported that ectopic endometrium was a larger IL6 producer compared with eutopic endometrium from the same patients. Nevertheless, Bergqvist *et al*. (2001) also reported a high IL6 level in secretory eutopic endometrium from endometriosis patients compared with normal endometrium using different methodologies (tissue homogenate protocol and IL6 commercial kit), IL6 content expression (pg/mg wet weight instead of pg/mg protein) and the secretory phase was not divided in early, mid and late stages as in the present study. Therefore, peritoneal activated macrophages and ectopic endometrial lesions are the main source of IL6, a molecule suggested to be involved in the pathogenesis of this disease (Guo 2007).

Endometriosis is associated with inflammation and pelvic pain; mediators such as cyclooxygenase (PTGS2), monocyte chemotactic peptide (CCL2) and matrix metalloproteinase (MMP9) are involved, molecules partially regulated by NFKB and steroid receptors. These three molecules were found in a high concentration at the lesions and peritoneal and menstrual fluids of women with endometriosis (Brenner *et al*. 2002, Hastings & Fazleabas 2006, Ulukus *et al*. 2006) probably mediated by the oestrogen hyper-responsiveness and

progesterone resistance in these patients. Thus, the peritoneal inflammatory environment induced by NFKB and the inhibition of apoptosis within eutopic endometrial cells transported from the uterus to the peritoneal cavity in patients with endometriosis may play an aetiological role in this pathology.

Differential gene expression is found by transcriptome or proteome studies in eutopic endometrium from women with and without endometriosis during the implantation window (Kao et al. 2003), the secretory phase (Zhang et al. 2006) or the late secretory endometrium (day 23–26; Sherwin et al. 2008). The altered genes found did not coincide in these three studies using different methodologies, showing that endometriosis is a multi-factorial disease with a wide clinical spectrum of patients that included wide pain and subfertility ranges, beside the dynamic and rapid changes experienced by the endometrium throughout the secretory phase of the menstrual cycle.

Finally, our results indicate that the NFKB system shows several anomalies in some proteins involved in its canonical activation pathway in eutopic endometrium from women with endometriosis that reduce its transcriptional function, affecting NFKBIA and IL6 expression, genes associated to proinflammatory and uterine reproductive processes. All these alterations occur mainly during the late secretory phase, the stage of the menstrual cycle where other anomalies related to the immune system and endometrial homeostasis have been reported for this disease. The pathogenesis of endometriosis remains unknown and several theories have been proposed to explain it. Retrograde menstruation is the most widely accepted theory (Sampson 1940), a phenomenon that occurs in the majority of healthy women, but endometriosis is developed in around 10% of women of reproductive age, suggesting that eutopic endometrium and a favourable peritoneal microenvironment may be involved in its aetiology.

Materials and Methods

Subjects

Eutopic endometrial tissue was obtained from 37 women undergoing laparoscopy for endometriosis associated with chronic pelvic pain, severe dysmenorrhoea or subfertility (one or no child), and from 42 eumenorrhoeic women undergoing laparoscopy for tubal sterilization (two to six children). The age of these women was 33.9 ± 4.4 years (range 23–42) and 35.5 ± 5.7 years (range 23–43) respectively ($P=0.17$). Both groups of patients had normal body mass index (BMI ≤ 25 kg/m^2) and they did not use any hormonal treatment for at least 3 months before surgery. Severity of endometriosis was assessed using the American Fertility Society staging system (mean score 35 ± 8, range 3–118). The Institutional Review Board approved this study and each patient signed a written informed consent. Endometrial samples were obtained by curettage from the uterine cavity

with a Pipelle suction curette and washed several times with ice-cold PBS to remove blood. The tissue was cut into slices and immediately frozen in liquid nitrogen for mRNA or protein preparation, IL6 determination or placed in 4% formalin/PBS (pH 7.2–7.4) for histological evaluation and immunohistochemistry.

Endometrial samples were dated according to Noyes criteria (Noyes et al. 1950) by an experienced histopathologist and classified as proliferative (days 5–14; 13 control and 10 endometriosis samples) or secretory endometria (days 15–28; 29 control and 27 endometriosis samples). The secretory phase was subdivided into early (days 15–18; seven control and seven endometriosis samples), mid (days 19–23; 12 control and 13 endometriosis samples) and late (days 24–28; ten control and seven endometriosis samples).

RNA preparation, cDNA synthesis and RT PCR

Total RNA was isolated from frozen proliferative and secretory endometria using RNA-solv Reagent (Omega Bio-Teck, Lilburn, GA, USA) plus glycogen (Chemicon International Inc., Temecula, CA, USA) and the purified pellet was resuspended in diethylpyrocarbonate-treated water. Complementary DNA (cDNA) was synthesized from 2 μg total RNA previously digested by DNase I (Fermentas AB, Vilnius, Lithuania) using random primers (Invitrogen) and 200 U RevertAid H Minus M-MuLV reverse transcriptase (Fermentas AB) following the manufacturer's instructions.

The amplification of RELA, CHUK, NFKBIA and IL6 mRNAs was assessed in duplicate using specific pairs of primers: RELA (308 bp, accession NM: 021975) upstream (260–279) 5′-TCA ATG GCT ACA CAG GAC CA-3′ and downstream (567–548) 5′-CAC TGT CAC CTG GAA GCA GA-3′; NFKBIA (376 bp, accession NM: 020529) upstream 5′-AAC CTG CAG CAG ACT CCA CT-3′ and downstream 5′-ACA CCA GGT CAG GAT TTT GC-3′ and IL6 (327 bp, accession NM: 0600) upstream (70–91) 5′-CCT TCT CCA CAA GCG CCT TC-3′ and downstream (396–376) 5′-GGC AAG TCT CCT CAT TGA ATC-3′. The amplification of CHUK mRNA was studied using specific pairs of primers as indicated in King et al. (2001). One microlitre cDNA per reaction was added to the reaction mix (total volume of 15 μl) containing PCR buffer, 3 mmol/l MgCl$_2$, 0.625 U Taq DNA polymerase (Invitrogen), 0.25 mmol/l nucleotide mix (Fermentas) and 0.4 μmol/l of each specific human primer (Invitrogen). The PCR was performed in a MyCycler thermal cycler (BioRad) at 94 °C for 60 s (denaturation), 55 °C for 45 s (annealing) and 72 °C for 90 s (extension), and repeated for 32 cycles. As an internal control, 18S rRNA cDNA (Schmittgen & Zakrajsck 2000) was amplified in each sample using the same conditions described above and repeated for 18 cycles. To determine that the amplification of all the genes was within a linear range, we previously evaluated the linearity of amplification of the corresponding transcripts in human endometrium explants and then the number of cycles was chosen.

Amplified fragment products were visualized on a 1.0% (w/v) agarose gel using ethidium bromide staining. Semi-quantification of PCR products was performed by image

analysis (Kodak EDAS 290 Electrophoresis Documentation and Analysis System, Kodak 1D Image Analysis Software).

Immunohistochemistry of RELA

Immunohistochemistry was performed as indicated in Johnson *et al*. (2004). Briefly, sections (4–6 µm thick) of human endometrial tissue at different stages of the menstrual cycle were deparaffinized in xylol and hydrated through decreasing grade alcohols. Sections were incubated in 10 mmol/l citrate buffer (pH 6.0) at 98 °C for 20 min. All slides were quenched in 3% H_2O_2 for 5 min at room temperature, blocked with 2% BSA in PBS (w/v) for 1 h at room temperature and then incubated with antibody against RELA (1:200; polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37 °C for 2 h. Immunodetection was performed by using the streptavidin–biotin–peroxidase system (LSAB 2, Dako Corp., Carpinteria, CA, USA), diaminobenzidine as chromogen and counterstained with haematoxylin. The immunohistochemical evaluation was performed by two blinded observers on at least 1000 cells in each sample calculating the percentage of RELA-positive cells. For negative control, the tissue was incubated with non-immune rabbit serum in place of the primary antibody.

Protein homogenate preparation

Frozen tissue was homogenized 1:5 (w/v) by a pellet pestle motor (PGC Scientifics, Frederick, MD, USA) in lysis buffer A (10 mmol/l Hepes (pH 7.9), 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DDT, 5 mmol/l NaF, 10 mmol/l $NaVO_4$, 10 mmol/l Na_3MoO_4 and 10 mmol/l KCl) in the presence of a protease inhibitor cocktail (Roche Applied Science), 0.5 mmol/l phenylmethylsulphonyl fluoride (PMSF) and 44.5 µg/ml trypsin inhibitor (Sigma Co). The homogenate was kept on ice for 15 min and Nonidet P-40 (0.05% v/v) was then added, mixed by vortexing and centrifuged at 10 000 ***g*** for 2 min at 4 °C. The supernatant contained the cytoplasmic protein extract. The pellet was suspended (1:1 w/v) in buffer B (20 mmol/l HEPES (pH 7.9), 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DDT, 5 mmol/l NaF, 10 mmol/l $NaVO_4$, 10 mmol/l Na_3MoO_4 and 400 mmol/l NaCl) in the presence of the protease inhibitor cocktail tablet and 0.5 mmol/l PMSF, sonicated 15 s at 4 °C, shook for 30 min on ice and centrifuged at 10 000 ***g*** for 1 min at 4 °C. The protein concentration was determined using the Bradford Assay reagent (BioRad).

Immunoblot analysis

Equal quantities (30 µg) of cytoplasmic or nuclear proteins were resolved in 7.5% SDS-PAGE and transferred to PVDF (BioRad) membrane. The membranes were blocked for 1 h in TTBS (20 mmol/l Tris–HCl (pH 7.5), 137 mmol/l NaCl and 0.1% (v/v) Tween 20) containing 3% (w/v) BSA. The membranes with cytoplasmic proteins were washed thrice for 15 min each time with TTBS and incubated with the primary antibodies: CHUK (1:300; polyclonal, Cell Signalling Technology Inc., Beverly, MA, USA), NFKBIA (1:300; polyclonal, Cell Signalling Technology) and p-NFKBIA (1:300; polyclonal, Cell Signalling Technology) for 24 h at 4 °C, and RELA (1:300;

polyclonal, Santa Cruz) and β-actin (1: 15 000, monoclonal, Sigma) for 1 h at 20 °C. The membranes with nuclear proteins were incubated with RELA antibody as indicated previously and GTF2B (1:500, monoclonal; BD Biosciences Pharmingen, Chicago, IL, USA) for 24 h at 4 °C. The membranes were washed as mentioned above, then incubated with anti-rabbit or anti-mouse IgG coupled to HRP (Amersham Biosciences) and visualized with chemiluminescence solution (0.1 mol/l Tris–HCl (pH 8.5); 2.5 mmol/l luminol, 0.4 mmol/l p-coumaric acid and 9% (v/v) H_2O_2, Sigma) and exposed to X-ray film. Semi-quantification of bands was performed by image analysis (Kodak 1D Image Analysis Software).

DNA shift assay

NFKB binding consensus oligonucleotide was obtained from Promega (Promega Co.) and end labelled with T_4 polynucleotide kinase (Invitrogen) using $[\gamma\text{-}^{32}P]ATP$ (Amersham Biosciences). Five microgram nuclear protein obtained as described above was added to the reaction mix (total volume of 20 µl) containing binding buffer (10 mmol/l Tris–HCl (pH 7.5), 5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1.25% (v/v) glycerol and 0.01% (v/v) Triton X100), 30 000 c.p.m. radiolabelled probe, 1 mmol/l dithiothreitol and 0.5 µg poly (dG-dC) (Amersham) and incubated at 4 °C for 60 min. Specific bands were verified with 100-fold excess of cold oligonucleotide. Samples were loaded on a 6% native polyacrylamide gel and run in non-denaturing Tris–borate–EDTA buffer (TBE). The gels were dried, and the radioactive bands were visualized with Typhoon 9200 (Amersham).

Determination of IL6 in explants

Twenty-five microlitre endometrium homogenate was used for IL6 determination according to the manufacturer's indications (Biotrak Easy ELISA, Amersham) and the reactions were measured in a microplate reader (BioRad model 680) at 450 and 630 nm as optional reference wavelength. Kit detection limit was 1.4 pg/ml and the reproducibility intra- and inter-assays were 6.2 and 7.0% respectively. The values were expressed as picogram of IL6 per milligram of protein.

Statistical analysis

Data were statistically analysed by Student's *t*-test or one-way ANOVA when comparing more than two categories such as the four stages of menstrual cycle, followed by Tukey's multiple comparison test. Differences were considered statistically significant when $P<0.05$. Results are expressed as mean ±s.e.m. The patients' ages are expressed as mean±s.d.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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