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Immunohistochemical study of the ubiquitin-nuclear factor-κB pathway in the endometrium of the baboon (papio anubis) with and without endometriosis

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Abstract

The aim of the present study was to conduct a semiquantitative immunohistochemical investigation into the levels of intermediary proteins within the nuclear factor (NF)-kappa B pathway throughout the menstrual cycle in a non-human primate, namely the baboon (Papio anubis), with and without endometriosis. Formalin-fixed eutopic (n = 2-4) and ectopic (n = 6-7) endometrial tissues from baboons at the mid-luteal phase were embedded in paraffin and examined for NF-kappa B pathway components (i.e. I kappa B kinase (IKK) alpha, IKK beta, phosphorylated (phospho-) I kappa B alpha and phospho-NF-kappa B p65 subunit), ubiquitin, 19S proteasome and the NF-kappa B activator tumour necrosis factor (TNF)-alpha. Similarly, endometrial tissues from baboons at the late follicular, mid-luteal and menses phase (n 2-4) were investigated to determine the levels of these proteins throughout the menstrual cycle. Cytoplasmic stromal IKK alpha and glandular 19S proteasome immunostaining was elevated in the ectopic endometrium, whereas levels of ubiquitin, phospho-p65, IKK beta, TNF-alpha and nuclear 19S proteasome were similar in the eutopic and ectopic endometrium. A significant decrease in phospho-I kappa B alpha nuclear immunostaining was observed within glandular cells of the ectopic endometrium. In the eutopic endometrium, IKK alpha, ubiquitin and 19S proteasome immunostaining was elevated in different phases of the menstrual cycle, whereas levels of phospho-p65, IKK beta, phospho-I kappa B alpha and TNF-alpha remained unchanged. We have demonstrated that, in the baboon endometriosis model, levels of IKK alpha immunostaining are elevated, whereas those of phospho-I kappa B alpha are reduced, consistent with the hypothesis that excessive NF-kappa B activity plays a role in reducing ectopic endometrial apoptosis, which contributes to the pathophysiology of endometriosis. Further studies are required to confirm a causal association between elevated IKK alpha levels and reduced endometrial apoptosis.

Keywords

anubis, papio, baboon, endometrium, pathway, endometriosis, without, factor, nuclear, ubiquitin, study, immunohistochemical, κb

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Immunohistochemical study of the ubiquitin–nuclear factor-κB pathway in the endometrium of the baboon (*Papio anubis*) with and without endometriosis

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Abstract. The aim of the present study was to conduct a semiquantitative immunohistochemical investigation into the levels of intermediary proteins within the nuclear factor (NF)-κB pathway throughout the menstrual cycle in a non-human primate, namely the baboon (*Papio anubis*), with and without endometriosis. Formalin-fixed eutopic ($n = 2$–4) and ectopic ($n = 6$–7) endometrial tissues from baboons at the mid-luteal phase were embedded in paraffin and examined for NF-κB pathway components (i.e. IκB kinase (IKK) α, IKKβ, phosphorylated (phospho-) IκBα and phospho-NF-κB p65 subunit), ubiquitin, 19S proteasome and the NF-κB activator tumour necrosis factor (TNF)-α. Similarly, endometrial tissues from baboons at the late follicular, mid-luteal and menses phase ($n = 2$–4) were investigated to determine the levels of these proteins throughout the menstrual cycle. Cytoplasmic stromal IKKα and glandular 19S proteasome immunostaining was elevated in the ectopic endometrium, whereas levels of ubiquitin, phospho-p65, IKKβ, TNF-α and nuclear 19S proteasome were similar in the eutopic and ectopic endometrium. A significant decrease in phospho-IκBα nuclear immunostaining was observed within glandular cells of the ectopic endometrium. In the eutopic endometrium, IKKα, ubiquitin and 19S proteasome immunostaining was elevated in different phases of the menstrual cycle, whereas levels of phospho-p65, IKKβ, phospho-IκBα and TNF-α remained unchanged. We have demonstrated that, in the baboon endometriosis model, levels of IKKα immunostaining are elevated, whereas those of phospho-IκBα are reduced, consistent with the hypothesis that excessive NF-κB activity plays a role in reducing ectopic endometrial apoptosis, which contributes to the pathophysiology of endometriosis. Further studies are required to confirm a causal association between elevated IKKα levels and reduced endometrial apoptosis.

Additional keyword: IκB kinase.

Introduction

Endometriosis is an oestrogen-dependent condition with an estimated prevalence of 10% in the general population (Olive and Schwartz 1993) that affects women of reproductive age. In endometriosis, endometrial glands and stroma from the eutopic endometrium are found in ectopic areas such as the ovaries, the posterior broad ligament, the anterior and/or posterior cul-de-sac and the uterosacral ligaments (Zeitvogel *et al.* 2001). Severe forms of endometriosis that cause chronic pain are seen in rectovaginal nodules due to the close apposition of lesions to nerves (Anaf *et al.* 2000). The associated pain and discomfort are exacerbated by peritoneal inflammation, deep infiltration, tissue damage, adhesion, fibrosis and the accumulation of menstrual blood that prohibits surrounding tissue movement. Endometriosis can be modelled in the baboon (*Papio anubis*) by transplantation of uterine fragments into the peritoneal cavity (Fazleabas 2006). This primate model provides an invaluable resource for understanding the pathogenesis of the condition because disease progression before and after transplantation can be determined. In addition, the baboon has a menstrual cycle that can produce spontaneous endometriosis (unlike rodents), it is phylogenetically closer to humans and it has reproductive anatomy and physiology similar to the human (Fazleabas *et al.* 2002).

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Dysregulation of endometrial cell survival is a key feature of endometriosis. In the human endometrium, the pleiotropic cytokine tumour necrosis factor (TNF)-α is one of many factors known to regulate cell survival. It is an activator of the nuclear factor (NF)-κB pathway, which drives the expression of survival-related genes, such as the baculoviral inhibitor of apoptosis protein repeat containing 2 (cIAP2; Nakanishi and Toi 2005). In addition, NF-κB induces pro-inflammatory cytokine transcription and the generation of reactive oxygen species (Yamauchi *et al.* 2004), as well as increasing the transcription of adhesion molecules and immune receptors (Schwartz and Ciechanover 1999). In the normal endometrium, transcription of TNF-α is regulated by NF-κB activation (Sakamoto *et al.* 2003). Paradoxically, TNF-α also activates apoptotic signalling through its Type 1 receptor (TNFR1; a member of the death receptor family), an effect that is counteracted by NF-κB. Excessive secretion of TNF-α, as found in some tumours (Wilson 2008) and ectopic endometrial lesions (Lee *et al.* 2008), or mutations in the NF-κB signalling pathway (Demchenko *et al.* 2010) can cause aberrant upregulation of NF-κB signalling and uncontrolled cell growth. Levels of apoptotic cells within the eutopic and ectopic endometrium of patients with endometriosis have been shown to be lower than in control patients (Gebel *et al.* 1998). TNF-α antagonists have been shown to inhibit NF-κB activation and prevent ectopic cell survival in endometriosis (Nakanishi and Toi 2005). Hence, it has been postulated that aberrant regulation of the NF-κB 'switch', possibly via excessive amounts of TNF-α, can contribute to ectopic cell survival seen in endometriosis (Lousse *et al.* 2008).

Several studies have investigated NF-κB signalling in the endometrium, including studies into NF-κB gene expression in normal endometrial tissues of women (King *et al.* 2001), mobility shift assays to determine its constitutive activity (González-Ramos *et al.* 2007) and studies in macrophages from the peritoneal fluid of women with and without the endometriosis (Cao *et al.* 2005; Lousse *et al.* 2008). Indeed, peritoneal macrophages in patients with endometriosis exhibit increased levels of NF-κB activation (Lousse *et al.* 2008) and are known to modulate the inflammatory and adherence mechanisms commonly associated with the condition (Dunselman *et al.* 2001). Several studies involving NF-κB inhibition and endometriosis have been published. Progesterone and its derivatives dienogest and danazol modulate NF-κB-mediated activation in endometriotic stromal cells (Horie *et al.* 2005). Human chorionic gonadotrophin (hCG) prevents the phosphorylation and degradation of IκBα, suppressing NF-κB nuclear translocation (Huber *et al.* 2007). BAY 11-7085, a soluble NF-κB inhibitor, has been shown to downregulate the expression of anti-apoptotic proteins Bcl-2 and Bcl-X_L while promoting expression of apoptotic proteins caspases-3, -8 and -9 in stromal cells isolated from women with endometriotic cysts (Nasu *et al.* 2007). Interestingly, the NF-κB inhibitor pyrrolidine dithiocarbonate (PDTC) and a proteasome inhibitor (bortezomib) have been used recently to successfully decrease the severity of induced peritoneal endometriosis in rats (Celik *et al.* 2008).

Ubiquitinylation plays a central role in regulating the magnitude and duration of NF-κB signalling (González-Ramos *et al.* 2007). In the classical role, ubiquitinylation of IκBα leads to its degradation within the 26S proteasome (Tansey 2004). This mechanism first requires receptor-activated phosphorylation of IκBα by the IκB kinase (IKK) complex and can occur via the morphogenetic-activated IKKα pathway (Hu *et al.* 1999) or via the pro-inflammatory IKKβ pathway (Delhase *et al.* 1999). Degradation of IκBα then frees the NF-κB subunits (e.g. p50, p65/RelA) to translocate to the nucleus (Sun and Chen 2004). More recently it has become clear that ubiquitination can both enhance and inhibit NF-κB signalling following activation of pro-inflammatory receptors such as TNFR1 (for a review, see Wullaert *et al.* 2006). Our previous study of ubiquitin in women with endometriosis revealed an association between altered cellular expression patterns of ubiquitin and cell survival (Ilad *et al.* 2004).

In the present study, using semiquantitative immunohistochemistry, we investigated levels of NF-κB-related proteins in eutopic and ectopic endometrial cells of baboons to provide evidence of aberrant NF-κB signalling in endometriosis with insight into candidate proteins for future cell survival studies involving endometriosis.

Materials and methods

Animals

Endometriosis was induced via laparoscopy and harvested using laparotomy via endometriectomy. Endometriotic lesions, between 1 and 16 months of the induced disease (Hastings and Fazleabas 2006), and eutopic endometrium from adult female baboons (*P. anubis*) were harvested. Control animals did not undergo multiple laparoscopies, but were subjected to laparotomies (Fazleabas *et al.* 2002; Hastings *et al.* 2006). The Animal Care Committee of the University of Illinois at Chicago granted ethical approval for all procedures conducted in this study. Between two and four eutopic tissues during late follicular (LF), mid-luteal (ML) and menstrual phases, as well as between six and seven ectopic endometrial tissues at ML, from baboons were investigated. This is consistent with previous studies (Gashaw *et al.* 2006; Jackson *et al.* 2007). Tissues were obtained from baboons between Days 9 and 11 after ovulation, which represents the window of uterine receptivity in this primate (Hastings and Fazleabas 2006).

Induction of endometriosis

A comprehensive description of the method by which endometriosis is induced in the baboon has been published elsewhere (Fazleabas *et al.* 2002). Menstrual endometrium (0.84 ± 0.22 g) was harvested on Day 2 of menses using a Unimar Pipelle (Cooper Surgical Inc., Shelton, CT, USA). Laparoscopy and video recording were used to confirm the absence of endometriotic lesions within the peritoneal cavity and the reproductive tract. Menstrual tissues in the Pipelle were guided via laparoscopy onto the pouch of Douglas, the ovaries and the broad ligament in close proximity to the ovaries. A second reseeding and laparoscopy was conducted during the succeeding menses. Upon the third menses, laparoscopy was again conducted to determine the development of endometriosis. Diagnostic laparoscopies were performed a second and third time, during the 4th and 10th months after principal inoculation. Lesion development was observed further using video recording and a

Table 1. Antibodies for immunohistochemistry
NF-κB, nuclear factor-κB; IKK, IκB kinase; TNF-α, tumour necrosis factor-α

Antigen	Supplier	Working dilution	Type
Ubiquitin	Dako (Botany, NSW, Australia)	1:400	Rabbit (polyclonal)
Phosphorylated NF-κB p65 (Ser^{536})	Cell Signalling (Beverly, MA, USA)	1:50	Rabbit (polyclonal)
IKKα (H-744)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	1:300	Rabbit (monoclonal)
(Human) TNF-α	Abcam (Cambridge, MA, USA)	1:200	Rabbit (polyclonal)
Proteasome 19S S1	Abcam	20 μg	Rabbit (polyclonal)
IKKβ (C-20)	Santa Cruz Biotechnology	1:200	Goat (monoclonal)
Phosphorylated IκBα (Ser^{32}/Ser^{36}; 5A5)	Cell Signalling	1:100	Mouse (monoclonal)

harmonic scalpel during laparoscopy. Biopsies of endometriotic tissues and sections of the surrounding normal peritoneum and eutopic endometrium were retrieved by Pipelle biopsy. The biopsies were subsequently snap-frozen in liquid nitrogen for future RNA extraction or fixed in buffered formalin for immunohistochemical investigation and analysis.

Immunohistochemistry

Formalin-fixed tissues were embedded in paraffin and cut into 5 μm sections, deparaffinised in xylene, rehydrated in a graded series of ethanol and boiled in antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for 5 min. Sections were immersed in peroxidase-blocking reagent (Dako, Botany Bay, NSW, Australia) for 10 min and incubated in a humidified chamber with blocking goat serum (Dako) for 30 min. Excess serum was drained and primary antibodies diluted in 1% normal serum (Table 1) were added to the sections. Sections were washed three times for 3 min each time with Tris-buffered saline (TBS) and kept in a humidified chamber overnight at 4°C. The primary antibodies used in the present study are listed in Table 1. Negative controls consisted of pre-immune serum to detect non-specific binding of protein at the same concentration as the immune serum or replacement of the primary antibody with normal goat IgG (Dako) or rabbit IgG (Dako) diluted at the same concentration as the primary antibody. Mouse ascites fluid was used as a control for phosphorylated IκBα (Sigma, St Louis, MO, USA). The antigen–antibody complex was detected using avidin–biotin complex–horse radish peroxidase (ABComplex/HRP; Dako, Botany Bay, Australia). 3,3-Diaminobenzidine (DAB) was used and sections were counterstained with haematoxylin, dehydrated in a graded series of ethanol and mounted.

Immunohistochemical grading of stained cells

The staining intensity of proteins within the nuclear and cytoplasmic compartment for the primary antibodies, as listed in Table 1, was evaluated in a semiquantitative fashion (i.e. scores of 0, 1, 2 or 3 corresponding to the presence of negative, weak, intermediate and strong brown staining, respectively). Using the method of Detre *et al.* (1995), nuclear and cytoplasmic protein expression from epithelial and stromal cells was evaluated separately and an 'H-SCORE' was calculated by taking the sum of the percentages of stained cells at different intensities and multiplying the value by the weighted intensity of the staining, specifically:

$$\text{H-SCORE} = \Sigma Pi\,(I + 1)$$

where I is the intensity score and Pi is the corresponding percentage of stained cells. Ten different areas were evaluated under a microscope for each slide (×400 magnification) and the percentage of cells at different staining intensities was determined in a blinded fashion by precoding the slides, ensuring that the identity of the baboon tissue being evaluated was concealed from the scorer. The average score from the 10 fields of view was used.

The average number of cells counted in our study for every 10 fields of view was 650 cells for eutopic glandular cell nuclei, 950 cells for ectopic glandular cell nuclei, 2090 cells for eutopic stromal cell nuclei and 2562 cells for ectopic stromal cell nuclei.

Determination of the H-SCORE for cytoplasmic and nuclear staining was conducted after image capture and analysed on a large computer screen where structures could be clearly delineated. Using this method, cytoplasmic components can be easily distinguished from nuclear staining.

Statistical analysis

JMP Macintosh Project (JMP) Statistical Discovery Software, 5.1.2 (SAS Institute Inc., Cary, NC, USA) was used for data analysis. All values are expressed as the mean ± s.e.m. All data were normally distributed. Student's *t*-test was used to determine differences in protein staining between eutopic and ectopic endometrium. For all analyses, $P < 0.05$ was considered significant.

Results

Comparison of protein expression within the eutopic and ectopic endometrium

Ubiquitin, TNF-α, IKKβ and phosphorylated p65 immunostaining

Intense nuclear ubiquitin staining was observed in all tissue types, with medium to strong cytoplasmic staining also seen (Table 2; Fig. 1). TNF-α immunostaining was mainly weak and restricted to the nucleus; minimal cytoplasmic staining was observed, except at menses, when some weak staining was observed (Table 3; Fig. 2). Faint nuclear IKKβ staining was seen in all tissues, accompanied by modest staining of the glandular cytoplasm (Table 4; Fig. 3). Low levels of nuclear phosphorylated (phospho-) p65 staining, together with weak to modest glandular

Table 2. Ubiquitin protein immunostaining in the eutopic and ectopic endometrium
Data are the mean ± s.e.m. *$P < 0.05$ compared with menses

	Ubiquitin H-SCORE			
	Nuclear		Cytoplasmic	
	Glands	Stroma	Glands	Stroma
Phase				
Menses	0	0	2.40 ± 2.40	4.45 ± 1.35
Late follicular	111.72 ± 7.68	124.66 ± 17.94*	8.20 ± 0.60*	8.30 ± 0.50
Mid-luteal	158.31 ± 17.94*	152.29 ± 5.73	7.87 ± 1.16*	7.70 ± 0.38
Eutopic *v.* ectopic endometrium				
Mid-luteal (eutopic endometrium)	158.31 ± 17.94	152.29 ± 5.73	7.87 ± 1.16	7.70 ± 0.38
Mid-luteal (ectopic endometrium)	149.01 ± 30.91	139.23 ± 12.42	7.45 ± 0.96	8.80 ± 1.21
P value	0.817	0.534	0.804	0.520

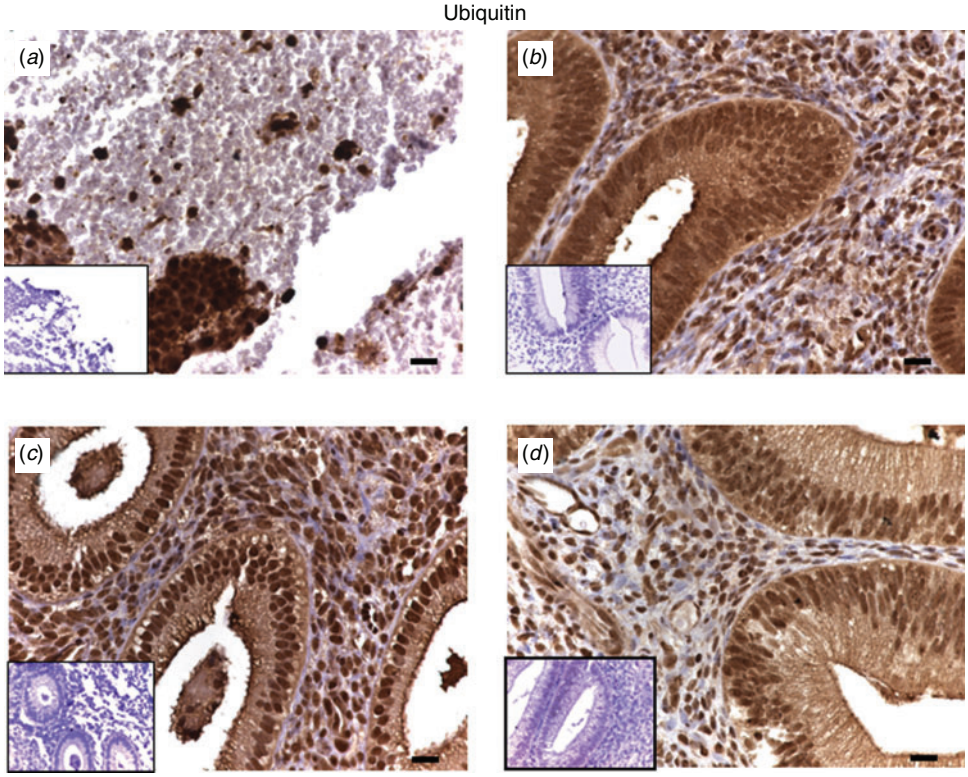

Fig. 1. Immunohistochemical staining of baboon endometrial sections for ubiquitin. (*a–c*) Eutopic endometrium at menses (*a*), the late follicular (*b*) and mid-luteal (*c*) phases. (*d*) Ectopic endometrium during the mid-luteal phase. Ubiquitin staining of both the nuclear and cytoplasmic compartments ranged from medium to high intensity. Scale bar = 20 μm.

cytoplasmic staining, was observed (Table 5; Fig. 4). There was no significant difference in the mean H-SCORE for ubiquitin (Table 2; Fig. 1), TNF-α (Table 3; Fig. 2), IKKβ (Table 4; Fig. 3) and phospho-p65 (Table 5; Fig. 4) within the nucleus and cytoplasm of the glands and stroma in the eutopic and ectopic endometrium.

19S proteasome immunostaining

Menses tissue exhibited strong 19S nuclear staining, accompanied by some weak to modest cytoplasmic staining (Table 6; Fig. 5). Eutopic tissues at LF and ML had weak nuclear and cytoplasmic staining that was located within glandular cells (Table 6; Fig. 5). Notably, the ectopic tissues exhibited cytoplasmic staining that was clearly seen within the glands (Table 6; Fig. 5). There was a significantly higher H-SCORE for 19S proteasome immunostaining within the cytoplasm of glands in the ectopic endometrium compared with the eutopic endometrium ($P = 0.04$; Table 6; Fig. 5). Levels of 19S immunostaining were not significantly different

Table 3. Tumour necrosis factor-α immunostaining in the eutopic and ectopic endometrium
Data are the mean ± s.e.m. *$P < 0.05$ compared with menses. TNF-α, tumour necrosis factor-α

	TNF-α H-SCORE			
	Nuclear		Cytoplasmic	
	Glands	Stroma	Glands	Stroma
Phase				
Menses	68.89 ± 68.89	100.85 ± 1.30	0.85 ± 0.35	2.05 ± 0.15
Late follicular	16.33 ± 14.78	18.83 ± 18.41	0	0
Mid-luteal	55.91 ± 21.63	31.77 ± 11.02	0.00	0*
Eutopic *v.* ectopic endometrium				
Mid-luteal (eutopic endometrium)	55.91 ± 21.63	31.77 ± 11.02	0.00	0
Mid-luteal (ectopic endometrium)	76.13 ± 20.58	65.71 ± 14.46	0.88 ± 0.54	0.95 ± 0.56
P value	0.569	0.10	0.180	0.158

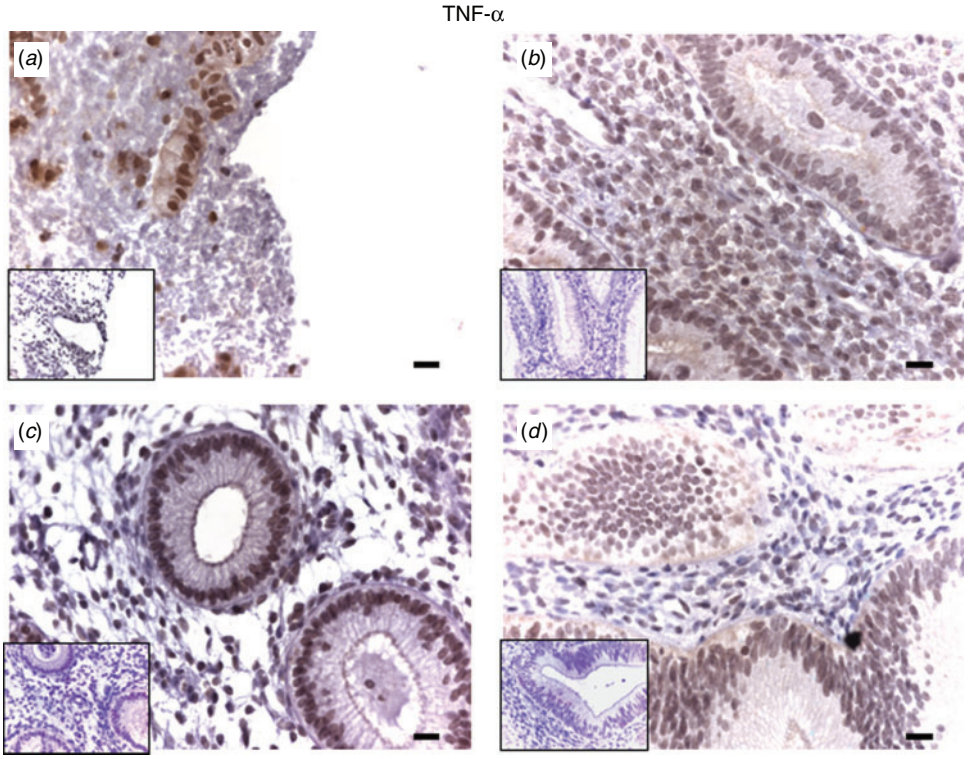

Fig. 2. Immunohistochemical staining of baboon endometrial sections for tumour necrosis factor (TNF)-α. (*a–c*) Eutopic endometrium at menses (*a*), the late follicular (*b*) and mid-luteal (*c*) phases. (*d*) Ectopic endometrium during the mid-luteal phase. Staining was weak in the nucleus and undetectable in the cytoplasm, except at menses when low intensity staining seen. Scale bar = 20 μm.

between the other cellular compartments in the eutopic and ectopic endometrium (Table 6; Fig. 5).

Immunostaining of phospho-IκBα

Minimal nuclear phospho-IκBα staining was observed in all tissue types (Table 7; Fig. 6). However, modest cytoplasmic staining was seen in the cytoplasm of glandular cells, particularly within discrete vesicular structures located in its basal layer, which are likely to be the Golgi apparatus (Schwartz *et al.* 2001; Table 7; Fig. 6). There was a significantly lower H-SCORE for phospho-IκBα staining within the nuclei of glands in the ectopic endometrium compared with the eutopic endometrium ($P = 0.045$; Table 7; Fig. 6). In the remainder of tissues, immunostaining for phospho-IκBα was not significantly different between the eutopic and ectopic endometrium (Table 7; Fig. 6).

Immunostaining for IKKα

Immunostaining for IKKα was observed in both nuclear and cytoplasmic compartments for all tissues types, with staining ranging from modest to strong depending on the tissue (Table 8; Fig. 7). Glandular cells were clearly stained throughout their structure (Table 8; Fig. 7). There was a significantly higher

Table 4. IκB kinase β immunostaining in the eutopic and ectopic endometrium
Data are the mean ± s.e.m. IKK, IκB kinase

Phase	IKKβ H-SCORE			
	Nuclear		Cytoplasmic	
	Glands	Stroma	Glands	Stroma
Phase				
Menses	0	0	3.20 ± 0.80	3.55 ± 1.85
Late follicular	0	0	3.30 ± 0.10	2.05 ± 0.15
Mid-luteal	0	0.26 ± 0.14	3.67 ± 0.57	2.87 ± 0.22
Eutopic *v.* ectopic endometrium				
Mid-luteal (eutopic endometrium)	0	0.26 ± 0.14	3.67 ± 0.57	2.87 ± 0.22
Mid-luteal (ectopic endometrium)	0.43 ± 0.43	1.22 ± 0.47	3.66 ± 0.42	3.01 ± 0.23
P value	0.601	0.296	0.990	0.830

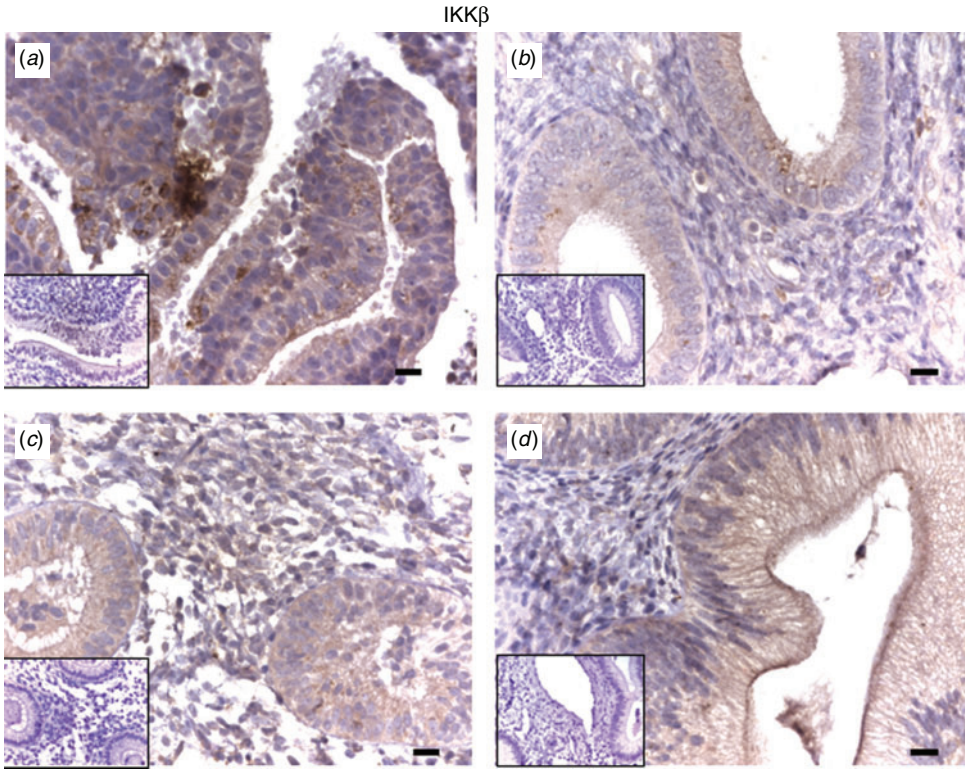

Fig. 3. Immunohistochemical staining of baboon endometrial sections for IκB kinase (IKK) β. (*a–c*) Eutopic endometrium at menses (*a*), the late follicular (*b*) and mid-luteal (*c*) phases. (*d*) Ectopic endometrium during the mid-luteal phase. IKKβ staining was only rarely detected in the nucleus and was weak in the glandular cytoplasm. Scale bar = 20 μm.

H-SCORE for IKKα staining within the cytoplasm of the stroma in the ectopic endometrium compared with the eutopic endometrium ($P = 0.015$; Table 8; Fig. 7).

Protein expression within the eutopic endometrium throughout the menstrual cycle

Immunostaining for IKKβ, phospho-IκBα and phospho-p65

There was no significant difference in the immunostaining for IKKβ (Table 4; Fig. 3), phospho-IκBα (Table 7; Fig. 6) and phospho-p65 (Table 5; Fig. 4) within the nucleus or cytoplasm of the glands and stroma throughout the menstrual cycle.

Ubiquitin immunostaining

A significantly higher H-SCORE for nuclear ubiquitin immunostaining was observed in the ML and menstrual phase ($P = 0.01$; Table 2; Fig. 1) and for cytoplasmic staining in the glands ($P = 0.03$; Table 2; Fig. 1). In addition, a significantly higher intensity of immunostaining for ubiquitin was seen in the

Table 5. Phosphorylated p65 nuclear factor-κB protein immunostaining in the eutopic and ectopic endometrium
Data are the mean ± s.e.m. NF-κB, nuclear factor-κB

	Phosphorylated p65 NF-κB H-SCORE			
	Nuclear		Cytoplasmic	
	Glands	Stroma	Glands	Stroma
Phase				
Menses	5.59 ± 5.59	6.77 ± 2.11	0.95 ± 0.75	0.30 ± 0.30
Late follicular	7.79 ± 4.47	4.11 ± 2.67	2.00 ± 0.10	2.00 ± 2.00
Mid-luteal	10.29 ± 8.72	5.46 ± 5.06	3.05 ± 0.55	0.28 ± 0.16
Eutopic *v.* ectopic endometrium				
Mid-luteal (eutopic endometrium)	10.29 ± 8.72	5.46 ± 5.06	3.05 ± 0.55	0.28 ± 0.16
Mid-luteal (ectopic endometrium)	25.42 ± 10.50	38.32 ± 14.11	4.63 ± 0.51	0.90 ± 0.44
P value	0.286	0.070	0.054	0.435

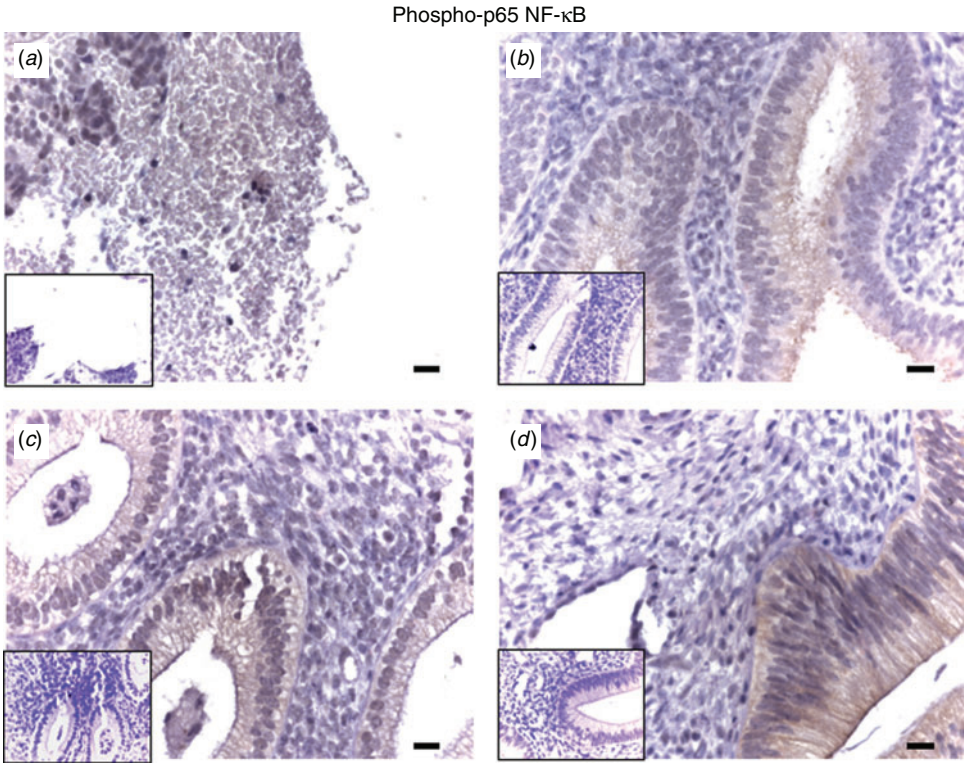

Fig. 4. Immunohistochemical staining of baboon endometrial sections with phosphorylated (phospho-) p65. (*a–c*) Eutopic endometrium at menses (*a*), the late follicular (*b*) and mid-luteal (*c*) phases. (*d*) Ectopic endometrium during the mid-luteal phase. Weak nuclear phospho-p65 staining was observed, with modest intensity staining seen within the glandular cytoplasm. Scale bar = 20 μm.

nucleus of stromal cells ($P = 0.04$; Table 2; Fig. 1) and the cytoplasm of glands ($P = 0.03$; Table 2; Fig. 1) at the LF phase compared with menses.

Immunostaining for TNF-α

A significant increase in TNF-α immunostaining within the cytoplasm of stromal cells was seen at menses ($P = 0.03$; Table 3; Fig. 2).

Immunostaining for 19S proteasome

Immunostaining for 19S proteasome during the menses phase was more intense within the nucleus of glands ($P = 0.015$) and stroma ($P = 0.0003$; Table 6; Fig. 5) compared with the ML phase. In stromal cells, a significant increase in the intensity of nuclear proteasomal immunostaining was also evident at menses compared with the LF phase ($P = 0.0005$; Table 6; Fig. 5). No significant differences in cytoplasmic proteasomal

Table 6. 19S proteasome protein immunostaining in the eutopic and ectopic endometrium
Data are the mean ± s.e.m. *$P < 0.05$ compared with menses. Bolded values indicate a significant difference

	19S proteasome H-SCORE			
	Nuclear		Cytoplasmic	
	Glands	Stroma	Glands	Stroma
Phase				
Menses	138.59 ± 11.41	178.00 ± 29.20	3.20 ± 0.40	3.95 ± 2.05
Late follicular	0	3.33 ± 3.33*	3.20 ± 0	0.75 ± 0.55
Mid-luteal	3.61 ± 3.11*	14.58 ± 8.42*	2.88 ± 0.97	2.15 ± 1.21
Eutopic *v.* ectopic endometrium				
Mid-luteal (eutopic endometrium)	3.61 ± 3.11	14.58 ± 8.42	2.88 ± 0.97	2.15 ± 1.21
Mid-luteal (ectopic endometrium)	43.07 ± 34.37	52.93 ± 19.37	4.93 ± 0.47	4.70 ± 1.02
P value	0.284	0.122	**0.040**	0.132

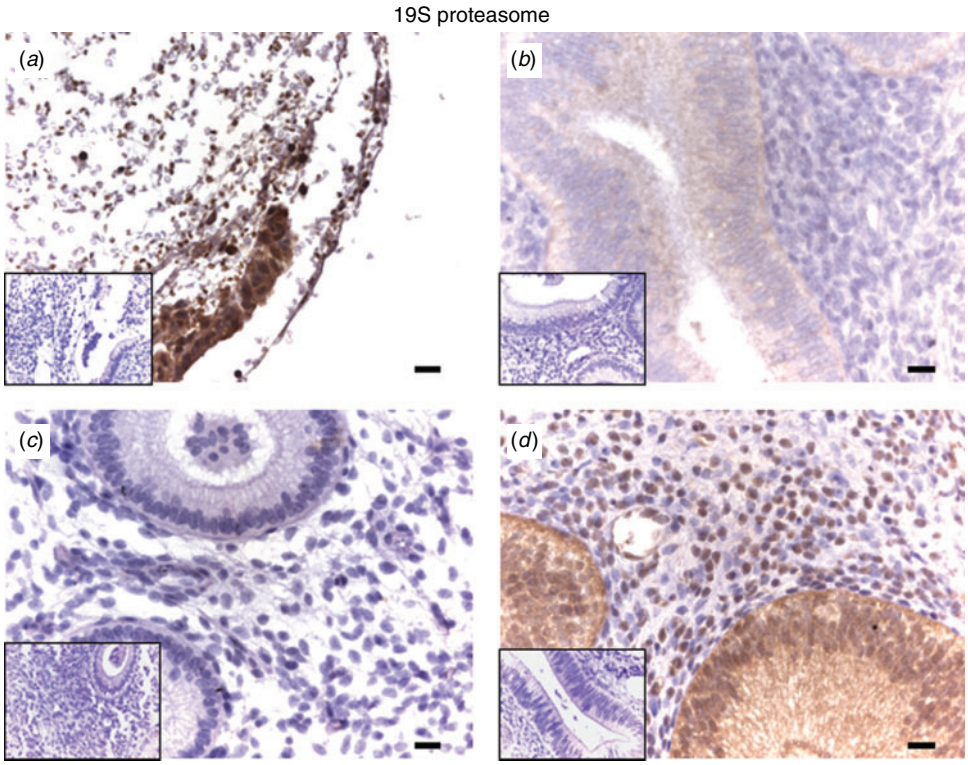

Fig. 5. Immunohistochemical staining of baboon endometrial sections for 19S proteasome. (*a–c*) Eutopic endometrium at menses (*a*), the late follicular (*b*) and mid-luteal (*c*) phases. (*d*) Ectopic endometrium during the mid-luteal phase. Nuclear 19S proteasome staining was evident at menses, together with modest intensity cytoplasmic staining. Eutopic tissues exhibited some weak nuclear and cytoplasmic 19S staining, particularly within glandular cells, whereas glands in ectopic tissues had a more distinct cytoplasmic staining. Scale bar = 20 μm.

immunostaining were found in either glandular or stromal cells throughout the menstrual cycle (Table 6; Fig. 5).

Immunostaining for IKKα

Immunostaining for IKKα in the cytoplasm of glands at the LF phase was more intense than at the ML phase ($P = 0.03$; Table 8; Fig. 7). This difference was also seen in the nucleus of stromal cells at menses compared with the LF ($P = 0.04$; Table 8; Fig. 7) and ML ($P = 0.03$; Table 8; Fig. 7) phases.

Discussion

The present study examined, for the first time, the expression and localisation of NF-κB-related proteins in the endometrium of the baboon (*P. anubis*). We found evidence of expression of

Table 7. Phosphorylated IκBα protein immunostaining in the eutopic and ectopic endometrium
Data are the mean ± s.e.m. Bolded values indicate a significant difference

	Phosphorylated IκBα H-SCORE			
	Nuclear		Cytoplasmic	
	Glands	Stroma	Glands	Stroma
Phase				
Menses	1.32 ± 1.32	8.90 ± 8.90	2.75 ± 1.25	0.80 ± 0.30
Late follicular	6.74 ± 6.74	5.02 ± 4.10	4.85 ± 1.25	1.00 ± 0.50
Mid-luteal	24.33 ± 9.28	13.91 ± 13.03	4.10 ± 0.97	0.40 ± 0.15
Eutopic *v.* ectopic endometrium				
Mid-luteal (eutopic endometrium)	24.33 ± 9.28	13.91 ± 13.03	4.10 ± 0.97	0.40 ± 0.15
Mid-luteal (ectopic endometrium)	6.51 ± 3.31	12.33 ± 6.10	3.87 ± 0.85	0.63 ± 0.26
P value	**0.045**	0.896	0.862	0.526

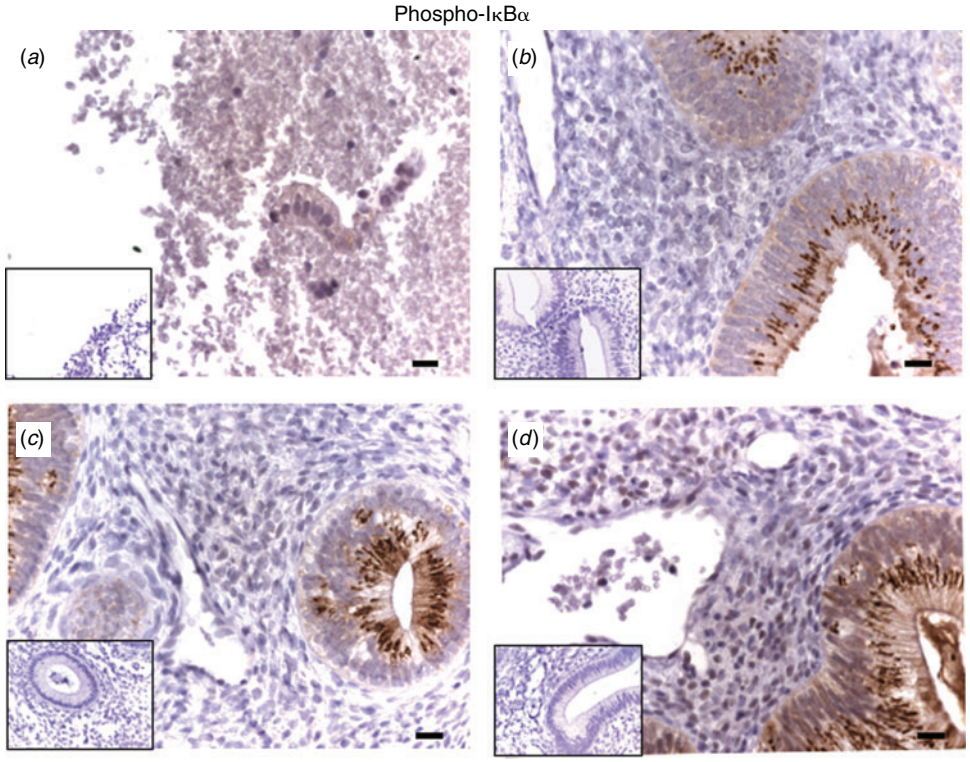

Fig. 6. Immunohistochemical staining of baboon endometrial sections for phosphorylated (phospho-) IκBα. (*a–c*) Eutopic endometrium at menses (*a*), the late follicular (*b*) and mid-luteal (*c*) phases. (*d*) Ectopic endometrium during the mid-luteal phase. Weak phospho-IκBα staining was occasionally seen in nuclear cells, whereas modest intensity cytoplasmic staining of discrete, vesicular structures was seen within the glandular basal layer. Scale bar = 20 μm.

all proteins studied, with varying levels of staining intensity according to tissue type and status. Although we found evidence of altered NF-κB signalling associated with endometriosis, the limitations of the present study do not allow us to make definitive conclusions regarding the functional consequences of these changes. Further studies are required to determine whether a causal relationships exists between altered NF-κB expression, endometrial cell survival and/or apoptosis and endometriosis.

Nevertheless, our observations do offer several interpretations regarding NF-κB signalling in endometrium. Firstly, IKKα, a cytoplasmic protein, is significantly elevated in the cytoplasm of stromal cells in endometriosis. It is possible that: (1) IKKα stays mainly in the cytoplasm after Iκβ phosphorylation, most likely bound to the IKK complex; (2) IKKα phosphorylates Iκβ on Ser^{32}/Ser^{36}, providing the trigger for Iκβ polyubiquitination, proteasome degradation and subsequent NF-κB release and activation of its target gene (Devin *et al.* 2000); (3) IKKα phosphorylates the p65 subunit of NF-κB on Ser^{536}, permitting NF-κB activation (Sakurai *et al.* 2003); and (4) IKKα is likely to contribute to increased cell survival in endometriosis at the ML phase.

Table 8. IκB kinase α protein immunostaining in the eutopic and ectopic endometrium

Data are the mean ± s.e.m. *$P < 0.05$ compared with menses; †$P < 0.05$ compared with the late follicular phase. Bolded values indicate a significant difference. IKK, IκB kinase

	IKKα H-SCORE			
	Nuclear		Cytoplasmic	
	Glands	Stroma	Glands	Stroma
Phase				
Menses	107.72 ± 82.52	117.75 ± 28.95	5.05 ± 1.75	5.50 ± 2.60
Late follicular	20.36 ± 9.77	24.02 ± 19.77*	7.60 ± 0	6.20 ± 0.50
Mid-luteal	21.14 ± 20.94	27.47 ± 24.62*	4.40 ± 0.63†	2.93 ± 0.38
Eutopic *v.* ectopic endometrium				
Mid-luteal (eutopic endometrium)	21.14 ± 20.94	27.47 ± 24.62	4.40 ± 0.63	2.93 ± 0.38
Mid-luteal (ectopic endometrium)	101.18 ± 23.18	72.51 ± 15.10	6.47 ± 0.61	6.32 ± 0.74
P value	0.063	0.117	0.056	**0.015**

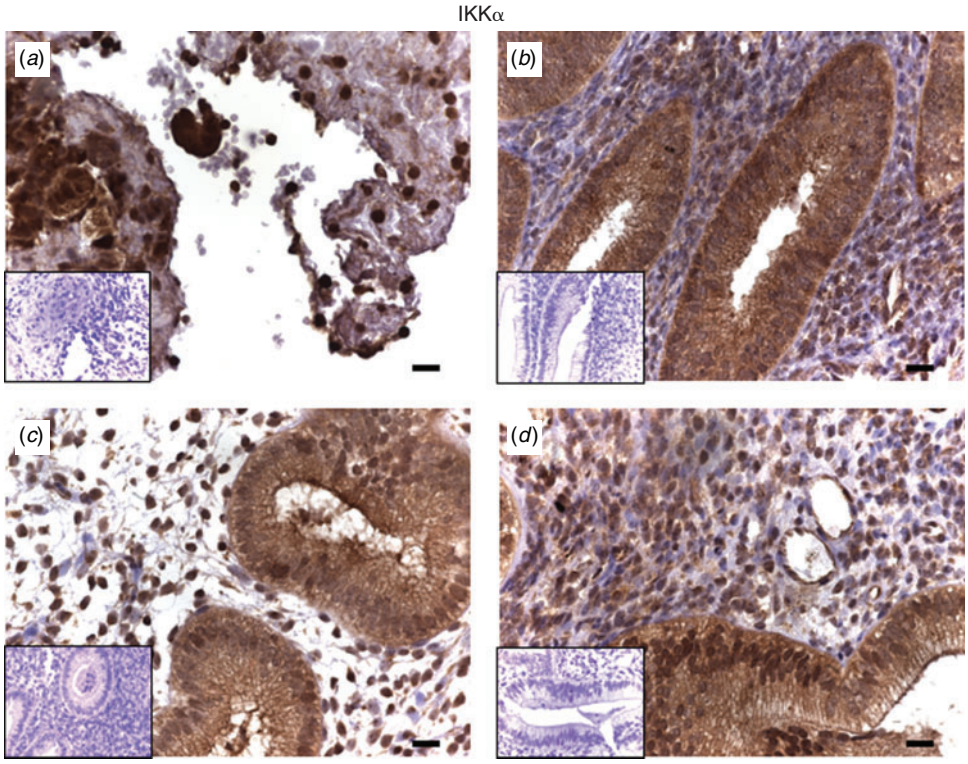

Fig. 7. Immunohistochemical staining of baboon endometrial sections for IκB kinase (IKK) α. (*a–c*) Eutopic endometrium at menses (*a*), the late follicular (*b*) and mid-luteal (*c*) phases. (*d*) Ectopic endometrium during the mid-luteal phase. Weak nuclear IKKα staining with more intense cytoplasmic staining was observed. Glandular cells were distinctly stained with anti-IKKα throughout their structure. Scale bar = 20 μm.

However, we could not rule out the possibility that an undefined activator of IKKα, such as the NF-κB-inducing kinase (NIK), preferentially phosphorylates p65 in the baboon (Ling *et al.* 1998).

Secondly, the significant increase in IKKα immunostaining in the cytoplasm of glands during the LF phase of the eutopic endometrium suggests an increased requirement for kinase activity, corresponding to the period of regeneration of the endometrium. Moreover, the elevated levels of IKKα immunostaining in nuclear stromal cells at menses may facilitate ectopic implantation. To our knowledge, this is the first time IKKα has been reported in the baboon endometrium; however, previous findings have shown nuclear staining of IKKα in the acinar and ductal cells of chronic pancreatitis (Farrow *et al.* 2004). Because chronic pancreatitis and menstruation are both associated with inflammatory mediators, the presence of IKKα in this tissue type may facilitate this function.

Another pathway that uses ubiquitin and IKKα to mediate NF-κB activation is the non-canonical pathway, which

predominantly has an anti-inflammatory and/or anti-apoptotic function. This pathway is activated by cytokines such as B cell-activating factor from the TNF family (BAFF) and CD40 ligand (CD40L) and is regulated by NIK and IKKα (not IKKβ and IKKγ), to cause NF-κB p100 subunit degradation to p52 (Ramakrishnan *et al.* 2004). However, the present study focused on TNF-α-mediated pro-inflammatory canonical activation of NF-κB because TNF-α is known to be commonly upregulated in the peritoneal fluid of women with endometriosis (Brieland *et al.* 2001) and can mediate protection against apoptosis via the NF-κB pathway (Nakanishi and Toi 2005).

The increase in 19S proteasome staining observed within the nucleus of glands and stroma at menses may indicate a greater level of nuclear protein recognition by the 19S protein during the time of ischaemic degeneration, whereby misfolded and short-lived proteins that are no longer required at menses are tagged by ubiquitin and discharged as menstrual effluent (Meiners *et al.* 2002). Proteasomal activity within the nucleus is upregulated during glucose starvation and hypoxic conditions, as seen in experiments using cancer cell lines (Kim *et al.* 1999; Ogiso *et al.* 1999). Previous immunohistochemical studies using fixed PtK2 (renal tissue) and L-132 (pulmonary epithelial) cells demonstrated proteasomes in the cytoplasm, the nucleus and cytoskeletal compartments (Palmer *et al.* 1994). Our endometrial cell staining also contains proteasomes in these regions. The proteasome is able to diffuse between the cytoplasm and the nucleus when the nuclear envelope is dispersed during metaphase (Reits *et al.* 1997).

The increase in glandular cytoplasmic 19S proteasome within ectopic endometrial cells at the ML phase suggests the possibility that more glandular cytoplasmic proteins are guided for proteasomal degradation in this tissue at this time. An example of such a protein is phospho-IκBα, staining of which was reduced in glandular nuclei in ML endometriotic tissues. The altered levels of this phosphoprotein could reflect increased proteasomal degradation, but also decreased phosphorylation or increased dephosphorylation (Sakurai *et al.* 2003). The biological significance of the changes would depend on which of these explanations was correct. Unfortunately, the immunohistochemical methodology used in the present study does not allow us to differentiate between them.

The comparable levels of phospho-IκBα and phospho-p65 NF-κB throughout the menstrual cycle, despite the trend towards increased TNF-α immunostaining within stromal cells of the endometrium at menses, suggests that NF-κB signalling may not be a major regulator of enhanced cell survival in the endometrium. This possibility is supported by the observed lack of difference in IKKβ staining between nuclear and cytoplasmic components of the glands and stroma within endometriotic and normal tissues. We originally postulated that a cytokine loop with increased NF-κB activation may lead to greater growth and survival of endometriotic cells in baboons (Chauhan *et al.* 2005). However, endometriotic stromal cells had levels of phopho-p65 NF-κB staining comparable to those in eutopic cells at the ML phase. Because there was no increase in phopho-p65 NF-κB immunostaining within eutopic stromal cells and because the ML phase correlates with the window of receptivity in the primate, eutopic cells are unlikely to create an environment conducive to ectopic endometrial cell implantation via the NF-κB pathway. However, it should be noted that, in the present study, we did not assess endometrial cell apoptosis or growth directly, so any conclusions regarding NF-κB activity and cell survival are purely speculative.

In conclusion, our findings suggest that IKKα may play a hitherto unappreciated role in endometrial cell survival signalling. Further studies, using manipulation of the NF-κB–IKK pathway, are required to establish the relationship between NF-κB signalling and enhanced cell survival associated with endometriosis.

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