Chapter 5
Immunohistochemical study of eutopic baboon endometrial tissues

5 Immunohistochemistry of eutopic baboon endometrial tissues

5.1 Introduction

In this study we investigated the role of the Ubiquitin-NFκβ pathway in endometrial cell progression throughout the menstrual cycle of the baboon. This is a complementary study to our examination of the ectopic endometrium (See Chapter 6).

As discussed in Chapter 1, IKK activation can occur via two separate mechanisms, one involving IKKα in the morphogenetic activated pathway (Hu *et al,* 1999) and the second involving IKKβ in the pro-inflammatory pathway (Delhase *et al,* 1999). In this study, we were interested in determining which pathway may be involved in endometrial cell progression and development in the endometrium of baboons.

5.2 Materials and Methods

5.2.1 Animals

2-4 baboon tissues obtained at varying cycle phases ranging from late follicular (LF), mid-luteal (ML) and menstrual phases were used for this study, as previously described in Section 2.1.

5.2.2 Immunohistochemistry

The ubiquitin, phospho NFκβ p65, IKKα, TNFα, 19S proteasome, IKKβ and phospho Iκβα proteins, were localised according to the method and recommended antibody dilutions in Section 2.1.3.

5.2.3 Immunohistochemical grading of stained cells

A semi-quantitative method was used to determine an individual H-SCORE for the nuclear and cytoplasmic compartments stained with the individual proteins as described in Section 2.1.4.

5.2.4 Statistical Analysis

P-values of < 0.05 were considered statistically significant and this was determined using calculations described in Section 2.1.5.

5.3 Results

5.3.1 IKKβ, Iκβα and NFκβ Immunostaining

There was no significant difference in the immunostaining for IKKβ (Table 5.1; Figure 5.1), Iκβα (Table 5.2; Figure 5.2) and NFκβ (Table 5.3, Figure 5.3) within the nucleus and cytoplasm of the glands and stroma throughout the menstrual cycle.

Table 5.1. IKK-β protein immunostaining in the endometrium.

Phase	**IKKβ H-Score**			
	Nuclear		***Cytoplasmic***	
	Glands	**Stroma**	**Glands**	**Stroma**
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
Menses	0	0	3.20 ± 0.80	3.55 ± 1.85
P-Value	NS	NS	NS	NS

NS = Not significant.

Table 5.2. Iκβα protein immunostaining in the endometrium.

Phase	**Iκβα H-Score**			
	Nuclear		***Cytoplasmic***	
	Glands	**Stroma**	**Glands**	**Stroma**
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
Menses	1.32 ±1.32	8.90 ± 8.90	2.75 ± 1.25	0.80 ± 0.30
P-Value	NS	NS	NS	NS

NS = Not significant

IKKB

IKBA

NFKB

Table 5.3. NFκβ protein immunostaining in the endometrium.

Phase	NFκβ H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
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
Menses	5.59 ± 5.59	6.77 ± 2.11	0.95 ± 0.75	0.30 ± 0.30
P-Value	NS	NS	NS	NS

NS = Not significant

5.3.2 Ubiquitin Immunostaining

A significantly higher immunostaining for ubiquitin immunostaining was observed between ML and menstrual phase in the nucleus (P =0.01; Table 5.4; Figure 5.4) and between cytoplasm of the glands (P = 0.03; Table 5.4; Figure 5.4). In addition, a significantly higher intensity of immunostaining for ubiquitin was seen in the nucleus of stromal cells (P =0.04; Table 5.1; Figure 5.1) and the cytoplasm of glands (P = 0.03; Table 5.4; Figure 5.4) at LF than at menses.

Table 5.4. Ubiquitin protein immunostaining in the endometrium.

Phase	UBIQUITIN H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
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
Menses	0	0	2.40 ± 2.40	4.45 ± 1.35
P-Value	**	*	* and **	NS

P < 0.05, *LF vs. Menses and**ML vs. Menses

NS = Not significant

ubiquitin

5.3.3 TNFα Immunostaining

A significant increase in TNFα immunostaining within the cytoplasm of stromal cells was seen at menses (P = 0.03; Table 5.5; Figure 5.5).

Table 5.5. TNF-α protein immunostaining in the endometrium.

Phase	TNF-α H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Late Follicular	16.33 ± 14.78	18.83 ± 18.41	0	0
Mid Luteal	55.91 ± 21.63	31.77 ± 11.02	0	0
Menses	68.89 ± 68.89	100.85 ± 1.30	0.85 ± 0.35	2.05 ± 0.15
P-Value	NS	NS	NS	**

P < 0.05, **ML vs. Menses

NS = Not significant

5.3.4 Proteasome Immunostaining

There was significantly higher immunostaining for the proteasome within the nucleus of glands (P = 0.015) and stroma (P = 0.0003; Table 5.6; Figure 5.6) during the menstrual phase than at the ML phase. Stromal cells also exhibited a significant increase in nuclear proteasomal immunostaining at menses than at the LF phase (P = 0.0005; Table 5.6; Figure 5.6). No significant difference in cytoplasmic proteasomal immunostaining was found in either glandular or stromal cells throughout the menstrual cycle (Table 5.6; Figure 5.6).

TNFa

Proteasome

Table 5.6. Proteasome protein immunostaining in the endometrium.

Phase	Proteasome H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
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
Menses	138.59 ± 11.41	178.00 ± 29.20	3.20 ± 0.40	3.95 ± 2.05
P-Value	**	* and **	NS	NS

P < 0.05, *LF vs. Menses, **ML vs. Menses

NS = Not significant

5.3.5 IKKα Immunostaining

A significantly higher immunostaining for IKKα was observed within the cytoplasm of glands at the LF phase than at the ML phase was seen (P = 0.03; Table 5.7, Figure 5.7). There was greater immunostaining in the nucleus of stromal cells at menses than at the LF (P = 0.04; Table 5.7, Figure 5.7) and ML phases (P = 0.03; Table 5.7, Figure 5.7).

Table 5.7. IKK-α protein immunostaining in the endometrium.

Phase	IKK-α H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
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
Menses	107.72 ± 82.52	117.75 ± 28.95	5.05 ± 1.75	5.50 ± 2.60
P-Value	NS	* and **	***	NS

P < 0.05, *LF vs. Menses, **ML vs. Menses and *** LF vs. ML

NS = Not significant

IKKa

5.4 Discussion

This study examines the intermediary proteins of the Ubiquitin-NFκβ pathway in the endometrium of the baboon (*Papio anubis*) and is, to our knowledge, the first of its kind. We have examined the role of the Ubiquitin-NFκβ pathway in ectopic endometrial cell survival in endometriosis of the baboon in a separate study. We also previously specifically looked at ubiquitin in women with endometriosis where we determined that ubiquitin was correlated with increased endometrial cell survival (Ilad *et al,* 2004). In this study, we collected endometrial tissue throughout the menstrual cycle at LF, ML and menstrual phases and investigated the level of IKKα immunostaining, as well as intermediary proteins in the Ubiquitin-NFκβ pathway.

An increase in 19S proteasome staining within the nucleus of glands and stroma at menses may indicate a greater level of nuclear protein recognition by the 19S protein, which is expected during this time of degeneration as a result of ischaemia. 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).

The significant increase in IKKα immunostaining in the cytoplasm of glands during the LF phase suggests an increased requirement for kinase activity during this phase, which corresponds to the period of regeneration of the endometrium. Moreover, the elevated levels of IKKα immunostaining in stromal cells at menses, during the phase where endometrial cells are most likely to be transported to ectopic locations via retrograde menstruation, may facilitate ectopic implantation in conditions such as endometriosis. Thus, it is possible that IKKα is a candidate for ectopic endometrial cell survival.

Despite the increased level of TNF-α immunostaining within stromal cells of the endometrium at menses, there were comparable levels of Iκβα, the endogenous inhibitor of NFκβ, as well as NFκβ itself throughout the menstrual cycle, which suggests that another protein pathway, or a group of pathways are responsible for greater endometrial cell survival. This is similar to the findings of our other study

comparing the eutopic and ectopic endometrium. An increase in TNF-α within stromal cells has been suggested to be a prelude to enhanced IL-8 cytokine levels that trigger NFκβ activation, which consequently enhance cytokine levels through a positive feedback mechanism (Yamauchi *et al,* 2004). The increase in cytokines has been shown to support cell growth and survival in multiple myeloma (Chauhan *et al,* 2005) but does not seem to be the mechanism involved the baboon endometrium. Moreover, the comparable level of 19S proteasome immunostaining within the cytoplasm of glands and stromal cells throughout the menstrual cycle suggests a similar substrate recognition activity. This is in preparation for protein degradation and/or unfolding to adequately fit into the 20S proteolytic chamber (Ciechanover 2005). Inhibitors of NFκβ such as Iκβα are usually degraded by the proteasome to allow NFκβ release and transcription of survival factors does not appear to be increased within the endometrium. This could allow continual Iκβα association with NFκβ, preventing a greater degree of cell survival through the NFκβ pathway (Nakanishi and Toi 2005).

Levels of ubiquitin immunostaining between the LF and ML phases within the glandular and stromal cell compartment are similar throughout the menstrual cycle. Our data is consistent with ubiquitin's potential role in supporting endometrial development should implantation occur, since an increased level at the ML versus menses phase exists, which corresponds to the period of increased receptivity in the baboon.

In conclusion, IKKα is a candidate for endometrial cell survival, but is likely to be modulated through a pathway other than Ubiqutin-NFκβ. The similar level in ubiquitin and NFκβ immunostaining suggests that ubiquitin may stabilize tagged proteins for various functions instead of proteasomal degradation, allowing Iκβα continual association with NFκβ, preventing the transcription of NFκβ survival factors. This result is similar to the mechanism seen in the ectopic endometrium, as observed in our other study (See Chapter 6).