Chapter 6
Immunohistochemical study of ectopic baboon endometrial tissues

6 Immunohistochemistry of ectopic baboon endometrial tissues

6.1 Introduction

The aim of the present study is to determine an intermediary protein in the Ubiquitin-NFκβ pathway that may be a candidate for ectopic endometrial cell survival in the non-human primate model of endometriosis the baboon (*Papio anubis*).

We examined the nuclear and cytoplasmic immunostaining of proteins involved in the NFκβ pathway from eutopic glandular and stromal cells at ML to determine their localisation and level of expression in Chapter 5 and now compare them with endometriotic tissues also at ML. We are interested to see if NFκβ is located in the cell nucleus of endometriotic tissues that would provide evidence that its anti-apoptotic activity may be involved in ectopic cell survival.

6.2 Materials and Methods

6.2.1 Animals

Between 2-7 eutopic and ectopic endometrium samples from ML phase baboons were included in this study. The protocol utilised for this chapter is listed in Section 2.1.

6.2.2 Immunohistochemistry

Immunohistochemical localisation of ubiquitin, phospho NFκβ p65, IKKα, TNFα, 19S proteasome, IKKβ and phospho Iκβα proteins was conducted using the protocol and optimised dilutions for antibodies described in Section 2.1.3.

6.2.3 Immunohistochemical grading of stained cells

The staining intensity of proteins within the nuclear and cytoplasmic compartment for the respective antibodies was evaluated using a semi-quantitative method as listed in Section 2.1.4, where an individual H-SCORE was determined.

6.2.4 Statistical analysis

All analyses are expressed as Mean ± SEM, with $P < 0.05$ considered statistically significant as determined by calculations at Section 2.1.5.

6.3 Results

6.3.1 Ubiquitin, TNF-α, IKKβ, NFκβ immunostaining

There was no significant difference in the mean H-Score for ubiquitin (Table 6.1, Figure 6.1), TNFα (Table 6.2, Figure 6.2), IKKβ (Table 6.3, Figure 6.3) and NFκβ (Table 6.4, Figure 6.4) within the nucleus and cytoplasm of the glands and stroma in the eutopic and ectopic endometrium.

Table 6.1: Ubiquitin protein immunostaining in ectopic [E] and eutopic endometrium.

Phase	**UBIQUITIN H-Score**			
	Nuclear		***Cytoplasmic***	
	Glands	**Stroma**	**Glands**	**Stroma**
Mid Luteal	158.31 ± 17.94	152.29 ± 5.73	7.87 ± 1.16	7.70 ± 0.38
Mid Luteal [E]	149.01 ± 30.91	139.23 ± 12.42	7.45 ± 0.96	8.80 ± 1.21
P-Value	0.817 (NS)	0.534 (NS)	0.804 (NS)	0.520 (NS)

NS = Not significant

Table 6.2: TNF-α protein immunostaining in ectopic [E] and eutopic endometrium.

Phase	**TNF-a H-Score**			
	Nuclear		***Cytoplasmic***	
	Glands	**Stroma**	**Glands**	**Stroma**
Mid Luteal	55.91 ± 21.63	31.77 ± 11.02	0.00	0
Mid Luteal [E]	76.13 ± 20.58	65.71 ± 14.46	0.88 ± 0.54	0.95 ± 0.56
P-Value	0.569 (NS)	0.10 (NS)	0.180 (NS)	0.158 (NS)

NS = Not significant

Table 6.3: IKK-β protein immunostaining in ectopic [E] and eutopic endometrium.

Phase	IKKβ H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Mid Luteal	0.00	0.26 ± 0.14	3.67 ± 0.57	2.87 ± 0.22
Mid Luteal [E]	0.43 ± 0.43	1.22 ± 0.47	3.66 ± 0.42	3.01 ± 0.23
P-Value	0.601 (NS)	0.296 (NS)	0.990 (NS)	0.830 (NS)

NS = Not significant

Table 6.4: NFκβ protein immunostaining in ectopic [E] and eutopic endometrium.

Phase	NFκβ H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Mid Luteal	10.29 ± 8.72	5.46 ± 5.06	3.05 ± 0.55	0.28 ± 0.16
Mid Luteal [E]	25.42 ± 10.50	38.32 ± 14.11	4.63 ± 0.51	0.90 ± 0.44
P-Value	0.286 (NS)	0.070 (NS)	0.054 (NS)	0.435 (NS)

NS = Not significant

Ubiquitin

TNF

IKKB

NFkb

6.3.2 Proteasome Immunostaining

There was a significantly higher H-Score for the proteasome within the cytoplasm of the glands in the ectopic endometrium in comparison with the eutopic endometrium (P = 0.04; Table 6.5; Figure 6.5). Immunostaining for the proteasome was not significantly different within the other cellular compartments in the eutopic and ectopic endometrium (Table 6.5; Figure 6.5).

Table 6.5: Proteasome protein immunostaining in ectopic [E] and eutopic endometrium.

Phase	**Proteasome H-Score**			
	Nuclear		***Cytoplasmic***	
	Glands	**Stroma**	**Glands**	**Stroma**
Mid Luteal	3.61 ± 3.11	14.58 ± 8.42	2.88 ± 0.97	2.15 ± 1.21
Mid Luteal [E]	43.07 ± 34.37	52.93 ± 19.37	4.93 ± 0.47	4.70 ± 1.02
P-Value	0.284 (NS)	0.122 (NS)	0.040 (*)	0.132 (NS)

*P < 0.05 and NS = Not significant.

Proteasome

6.3.3 Iκβα immunostaining

There was a significantly lower H-score for Iκβα within the nuclei of the glands in the ectopic endometrium compared with the eutopic endometrium (P = 0.045; Table 6.6; Figure 6.6). Immunostaining for Iκβα was not significantly different within the other cellular compartments in the eutopic and ectopic endometrium (Table 6.6; Figure 6.6).

Table 6.6: Iκβα protein immunostaining in ectopic [E] and eutopic endometrium.

Phase	IκβαH-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Mid Luteal	24.33 ± 9.28	13.91 ± 13.03	4.10 ± 0.97	0.40 ± 0.15
Mid Luteal [E]	6.51 ± 3.31	12.33 ± 6.10	3.87 ± 0.85	0.63 ± 0.26
P-Value	0.045 (*)	0.896 (NS)	0.862 (NS)	0.526 (NS)

*P < 0.05 and NS = Not significant.

6.3.4 IKKα immunostaining

There was a significantly higher H-Score for IKKα within the cytoplasm of the stroma in the ectopic endometrium compared with the eutopic endometrium (P = 0.015; Table 6.7, Figure 6.7). Immunostaining for IKKα was not significantly different within the other cellular compartments of the eutopic and ectopic endometrium.

Table 6.7: IKK-α protein immunostaining in ectopic [E] and eutopic endometrium.

Phase	IKK-α H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Mid Luteal	21.14 ± 20.94	27.47 ± 24.62	4.40 ± 0.63	2.93 ± 0.38
Mid Luteal [E]	101.18 ± 23.18	72.51 ± 15.10	6.47 ± 0.61	6.32 ± 0.74
P-Value	0.063 (NS)	0.117 (NS)	0.056 (NS)	0.015 (*)

*P < 0.05 and NS = Not significant.

IKBA

IKKA

6.4 Discussion

The 19S proteasome was chosen for immunostaining, as a functioning 19S gives us an indication that an unfolded polypeptide ready to be degraded can be recognized and can pass through a narrow entrance and be presented to the 20S component for intracellular degradation (Dolenc *et al*., 1998). Without a 19S subunit, there is no possibility for a protein to be recognized and guided for degradation, despite the presence of a functioning 20S catalytic protein.

19S proteasome staining is elevated in the glandular cytoplasm of endometriosis tissues, indicating that a greater level of protein recognition by the 19S protein is needed. There is the possibility that more glandular cytoplasmic proteins are being guided by the 19S proteasome for potential degradation within the 20S chamber.

The Iκβα antibody used in this study detects phosphorylated Iκβα at Serine 32/Serine 36. Thus the significantly reduced glandular nuclear phosphorylated Iκβα mean H-Score evident in endometriotic tissues, compared to the eutopic endometrium, may be attributed to a greater phosphatase activity, which can quickly dephosphorylate Ser-536 (Sakurai *et al,* 2003). This can potentially decrease the amount of Iκβα proteasomal degradation, possibly allowing Iκβα's continual association with NF-κβ. Thus another protein and not NFκβ is possibly responsible for glandular cell survival in the baboon endometriosis model, especially as NFκβ is unlikely to translocate to transcribe survival factors, for there is an absence of NF-κβ activity within the nucleus (Nakanishi and Toi 2005).

IKKα, a cytoplasmic protein, is significantly elevated in the cytoplasm of stromal cells in endometriosis. It is therefore possible that: 1) IKKα stays mainly in the cytoplasm after Iκβ phosphorylation, most likely bound to the IKK complex; 2) IKKα phosphorylates Iκβ on Ser32/Ser 36, providing the trigger for Iκβ polyubiquitination, proteasome degradation and subsequent NFκβ release and activation of its target gene

(Devin *et al,* 2000); 3) IKKα phosphorylates the p65 subunit of NFκβ on Ser-536, permitting NFκβ activation (Sakurai *et al,* 2003) and 4) IKKα is likely to contribute to increased cell survival in endometriosis at ML phase. However, we could not rule out the possibility that an undefined activator of IKKα, such as the nuclear factor kappa beta inducing kinase (NIK), preferentially phosphorylates p65 in the baboon (Ling *et al,* 1998).

Our data shows no difference in IKKβ staining between nuclear and cytoplasmic components of glands and stroma within endometriotic and normal tissues, illustrating that IKKβ may behave similarly within the eutopic and ectopic endometrium. IKKβ doesn't appear to play a role in Iκβα phosphorylation in the baboon endometriosis model and is not likely to be responsible for ectopic endometrial cell survival. The kinase predominantly resides within the cytoplasm and is not commonly found in the nucleus.

Stromal cells express IL-8, after TNF-α activation, which leads to NFκβ expression (Yamauchi *et al,* 2004). NFκβ is also a known enhancer of IL-6 and IL-8 cytokine secretion, upon TNF-α activation (Roger *et al,* 2004). This positive feedback mechanism may subsequently lead to greater growth and survival of endometriotic cells in baboons (Chauhan *et al,* 2005). Endometriotic stromal cells had comparable levels of NFκβ staining to eutopic cells at ML phase. Since there was no increase in NFκβ immunostaining within eutopic stromal cells and as the ML phase correlates to the window of receptivity in the primate, eutopic cells are unlikely to create an environment conducive to ectopic endometrial cell implantation upon menstrual reflux via the NFκβ pathway. NFκβ is possibly kept in the cytoplasm at comparable levels by the endogenous inhibitor Iκβ.

The similar levels of ubiquitin and NFκβ immunostaining within eutopic and endometriotic tissues of baboons suggest that ubiquitin may stabilize tagged proteins for various functions instead of proteasomal degradation that would otherwise allow

NFκβ to dissociate from Iκβα and to initiate the transcription of survival factors. Therefore, it is thus likely that a pathway other than NFκβ is responsible for endometriosis in baboons. Future studies may look at the c-Jun N-terminal kinase (JNK) pathway, which also responds to TNF-α mediated activation and can activate IKK-α upstream, whilst causing cytokine and immunoregulatory response protein transcription within the nucleus. In conclusion, we demonstrated that IKKα kinase immunostaining is elevated in the baboon endometriosis model and is a possible candidate for ectopic endometrial cell survival.