Chapter 8
Immunohistochemical study of ectopic human endometrial tissues

8 Immunohistochemistry of ectopic human endometrial tissues

8.1 Introduction

Similar to our investigation of the intermediary proteins (IKKα, IKKβ, Iκβα and proteasome) in the eutopic and ectopic endometrial tissues in the baboon in Chapter 5 and 6, we investigated the same proteins in tissues derived from humans. Archived formalin fixed and paraffin embedded tissues from humans at proliferative and secretory phases was examined. Our data suggests a potentially different 19S proteasome regulatory activity within the nucleus of ectopic stromal cells during the proliferative and secretory phase. In addition, the 19S proteasome protein recognition capability within the cytoplasmic compartment may be lower within the ectopic endometrium. Our data reveals neither IKKα nor IKKβ would have a greater phosphorylation potential to enable the activation of NFκβ in women with endometriosis, particularly as there are no significant changes in Iκβα levels between tissue types and that since NFκβ protein is absent.

The same proteins involved in the ubiquitin mediated NFκβ pathway as listed in Table 2.2 were also studied in the eutopic and ectopic endometrial tissues of women, by following the underlying principles in Section 6.2. Similar to the discussion in Section 7.2, we did not investigate ubiquitin in this patient cohort, as we had published those results previously (Ilad *et al,* 2004). In addition, we excluded the examination of menstrual phase tissues and TNF-α protein staining, as described in Section 7.2, contrasting with the study in Chapter 6.

8.2 Materials and Methods

8.2.1 Human tissues

19 endometrial tissues and 9 endometriotic tissues were used for this study, as previously described in Section 2.2.1. For the eutopic tissues, 9 proliferative and 10 secretory phase samples were obtained, whilst 5 proliferative and 4 secretory phase

tissues were sectioned for ectopic tissues. We sought the expertise of the resident hospital statistician, Dr Karen Byth, to help us determine the minimum number of patients required to provide a statistically viable study utilising immunohistochemistry. Based on our previous study, where we examined ubiquitin in 59 endometrial tissues and 20 endometriotic tissues respectively, Dr Byth has determined that a sample size of 6 in both the proliferative and secretory phases, will have an 80% power to detect a difference in means between the endometrial tissues, assuming that the common standard deviation is 0.150, using a two group t-test with a 0.050 two-sided significance level, as described in Section 7.4.1. In addition, 4 patients in both the proliferative and secretory phases in endometriotic tissues is sufficient to identify a difference in means with the same power and rationale as above, assuming that the common standard deviation is 0.090.

As discussed in Section 7.4.1, ubiquitin mediated tagging of IKK and Iκβα for proteasomal degradation control the NFκβ pathway. By knowing the minimum number of patients required for immunohistochemistry, we should be able to ascertain how the levels of proteins tagged by ubiquitin in the NFκβ pathway differs between the eutopic and ectopic endometrial tissues of women.

8.2.2 Immunohistochemistry

Immunohistochemical localisation of phospho NFκβ p65, IKKα, 19S proteasome, IKKβ and phospho Iκβα proteins was conducted using the protocol and optimised dilutions for antibodies in Table 2.2.

8.2.3 Immunohistochemical grading of stained cells

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

8.2.4 Statistical Analysis

All analyses were expressed as Mean ± SEM, with $P < 0.05$ considered statistically significant as determined by the same calculations mentioned for baboons in Section 2.1.5.

8.3 Results

8.3.1 IKKβ, NFκβ, 19S proteasome, Iκβα and IKKα immunostaining in proliferative phase

There was no significant difference in the mean H-Score for IKKβ (Table 8.1; Figure. 8.1), NFκβ (Table 8.3; Figure. 8.3), Iκβα (Table 8.7; Figure. 8.7) and IKKα (Table 8.9; Figure. 8.9), within the nucleus and cytoplasm of the glands and stroma of the eutopic and ectopic endometrium.

However, a significant increase in 19S proteasome staining exists within the nucleus of ectopic stromal cells in comparison to the eutopic endometrium ($P = 0.02$; Table 5; Figure. 5).

8.3.2 IKKβ, NFκβ, 19S proteasome, Iκβα and IKKα immunostaining in secretory phase

Immunostaining for NFκβ (Table 8.4; Figure 8.4), Iκβα (Table 8.8; Figure8.8) and IKKα (Table 8.10; Figure 8.10) was not significantly different in each of the cellular compartments of the eutopic and ectopic endometrium.

An absence of IKKβ within the cytoplasmic compartment of glandular cells of the ectopic endometrium during secretory phase was seen in comparison to the eutopic endometrium ($P = 0.009$; Table 8.2; Figure 8.2).

There was a significantly lower H-Score for the 19S proteasome within the nucleus of stromal cells and cytoplasmic compartment of glandular cells of the ectopic

endometrium, in comparison with the eutopic endometrium during the secretory phase (P = 0.04; Table 8.6; Figure. 8.6) and (P = 0.02; Table 8.6; Figure. 8.6) respectively.

Table 8.1: IKK-β protein immunostaining in ectopic [E] and eutopic endometrium during the proliferative phase.

Phase	IKKβ H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Proliferative	2.10 ± 1.42	0.09 ± 0.06	0.46 ± 0.35	0.28 ± 0.21
Proliferative [E]	15.51 ± 12.83	76.24 ± 74.60	0	2.03 ± 1.99
P-Value	0.148 (NS)	0.154 (NS)	0.092 (NS)	0.190 (NS)

NS = Not significant

Table 8.2: IKK-β protein immunostaining in ectopic [E] and eutopic endometrium during the secretory phase.

Phase	IKKβ H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Secretory	6.95 ± 6.81	2.11 ± 1.54	0.77 ± 0.32	0.42 ± 0.23
Secretory [E]	0	2.03 ± 1.37	0	0.08 ± 0.08
P-Value	0.154 (NS)	0.479 (NS)	0.009 (*)	0.068 (NS)

*P < 0.05 and NS = Not significant

IKKBPro

IKKBSec

Table 8.3: NFκβ protein immunostaining in ectopic [E] and eutopic endometrium during the proliferative phase.

Phase	**NFκβ H-Score**			
	Nuclear		***Cytoplasmic***	
	Glands	**Stroma**	**Glands**	**Stroma**
Proliferative	0	0	0	0
Proliferative [E]	0	0	0	0
P-Value	N/A	N/A	N/A	N/A

N/A = Not applicable

Table 8.4: NFκβ protein immunostaining in ectopic [E] and eutopic endometrium during the secretory phase.

Phase	**NFκβ H-Score**			
	Nuclear		***Cytoplasmic***	
	Glands	**Stroma**	**Glands**	**Stroma**
Secretory	0	0	0	0.04 ± 0.04
Secretory [E]	0	0	0	0
P-Value	N/A	N/A	N/A	0.159 (NS)

N/A = Not applicable; NS = Not significant

NFKBPro

NFKBSec

Table 8.5: Proteasome protein immunostaining in eutopic [E] and ectopic endometrium during the proliferative phase.

Phase	PROTEASOME H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Proliferative	2.76 ± 2.32	0.21 ± 0.21	3.65 ± 1.06	1.20 ± 0.75
Proliferative [E]	13.07 ± 12.34	23.31 ± 11.51	1.60 ± 1.22	1.51 ± 0.97
P-Value	0.20 (NS)	0.02 (*)	0.95 (NS)	0.38 (NS)

*P < 0.05 and NS = Not significant

Table 8.6: Proteasome protein immunostaining in eutopic [E] and ectopic endometrium during the secretory phase.

Phase	PROTEASOME H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Secretory	60.76 ± 40.62	25.40 ± 13.22	3.33 ± 0.93	1.79 ± 0.54
Secretory [E]	5.22 ± 5.22	2.32 ± 2.32	1.31 ± 1.31	2.58 ± 1.92
P-Value	0.09 (NS)	0.04 (*)	0.02 (*)	0.93 (NS)

*P < 0.05 and NS = Not significant.

ProteasomePro

ProteaseomSec

Table 8.7: Iκβα protein immunostaining in ectopic [E] and eutopic endometrium during the proliferative phase.

Phase	IκβαH-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Proliferative	0	0.57 ± 0.56	0.03 ± 0.02	0
Proliferative [E]	1.13 ± 1.13	0.10 ± 0.10	0.18 ± 0.18	0.60 ± 0.60
P-Value	0.159 (NS)	0.200 (NS)	0.195 (NS)	0.159 (NS)

NS = Not significant.

Table 8.8: Iκβα protein immunostaining in ectopic [E] and eutopic endometrium during the secretory phase.

Phase	IκβαH-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Secretory	0	0.02 ± 0.02	0.29 ± 0.21	0.01 ± 0.01
Secretory [E]	0	1.90 ± 1.67	0	0.42 ± 0.42
P-Value	N/A	0.131 (NS)	0.079 (NS)	0.165 (NS)

N/A = Not applicable as no P-value can be calculated and NS = Not significant.

IKBaPro

IKBaSec

Table 8.9: IKK-α protein immunostaining in ectopic [E] and eutopic endometrium during the proliferative phase.

Phase	IKK-α H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Proliferative	0.09 ± 0.09	2.58 ± 2.43	0.13 ± 0.11	0.16 ± 0.09
Proliferative [E]	0	0	0.30 ± 0.30	0
P-Value	0.159 (NS)	0.145 (NS)	0.936 (NS)	0.035 (NS)

NS = Not significant.

Table 8.10: IKK-α protein immunostaining in ectopic [E] and eutopic endometrium during the secretory phases.

Phase	IKK-α H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Secretory	0	0	0.04 ± 0.04	0.06 ± 0.04
Secretory [E]	0	0	0.20 ± 0.20	0.05 ± 0.05
P-Value	N/A	N/A	0.218	0.588

N/A = Not applicable as no P-value can be calculated and NS = Not significant.

IKKaPro

IKKaSec

8.4 Discussion

The 19S proteasome staining is higher in the nucleus of ectopic stromal cells in comparison to the eutopic endometrium during proliferative phase. This indicates that a higher level of recognition by the 19S protein is needed and guided to the 20S chamber for degradation. This contrasts with the finding in the nucleus of stromal cells and glandular cytoplasm of endometriosis tissues during secretory phase where a lower 19S protein level exists. Thus a lower level of protein recognition by the 19S protein is needed and guided to the 20S chamber for degradation in these cellular compartments.

Our data suggests that neither IKKα nor IKKβ would have a greater phosphorylating potential of the p65 subunit of NFκβ on Ser-536. Furthermore, IKKβ is definitively not the kinase involved in phosphorylating Ser-536 on NFκβ in endometriosis, as this was absent within the ectopic tissue during secretory phase. This result was statistically significant in comparison to the eutopic endometrium.

The similar levels of Iκβα and the absence of NFκβ immunostaining within eutopic and endometriotic tissues indicate that the NFκβ pathway is not responsible for any potential survival potential of endometriotic tissues in our patient cohort.