Chapter 7
Immunohistochemical study of eutopic human endometrial tissues

7 Immunohistochemical study of eutopic human endometrial tissues

7.1 Introduction

To complement our immunohistochemical study in the baboon, the same proteins listed in Table 2.2 were also used in the eutopic endometrial tissues of women, by investigating proteins within the ubiquitin mediated NFκβ pathway, following the rationale described in Section 5.2. However, we did not look at the protein ubiquitin in this patient cohort, as we had previously described and published those results (Ilad *et al,* 2004). In addition, we excluded menstrual phase tissues and TNF-α protein staining. This contrasts the methodology in Chapter 5 for baboons, whereby late follicular, mid luteal and menses endometrial tissues were examined and TNF-α was included in the study. We conducted numerous titre and protocol optimisations before a final antibody concentration was derived for all the proteins, reducing reagents such as avidin and biotin, which are important for blocking endogenous biotin.

A decision was then made to investigate downstream proteins in the ubiquitin mediated NFκβ pathway in proliferative and secretory phase tissues, as this was likely to give us an indication whether or not NFκβ was potentially involved in the regeneration of the endometrium during the menstrual cycle. We sought the expertise of the resident hospital statistician, Dr Karen Byth, to determine the minimum number of patients required to give a statistically valid analysis of proteins tagged by ubiquitin. Since the analysis was based on our previous study of ubiquitin stained during proliferative and secretory phase tissues, we were able to determine the minimum number of patients required for these two phases. However, we were unable to extrapolate the same rationale for the menstrual phase tissues. The fact that we could also not purchase extra reagents that would have enabled us to investigate the protein levels in additional tissues, we therefore decided not to investigate the menses phase in humans.

7.2 Materials and methods

7.2.1 Human tissues

19 endometrial tissues (9 proliferative and 10 secretory phase) were examined and prepared as previously described in Section 2.2. Based on our previous study, where we examined ubiquitin in 59 eutopic endometrial tissues (proliferative and secretory phases), Dr Byth determined that a sample size of 6 in both phases will have an 80% power to detect a difference in means, assuming that the common standard deviation is 0.150, using a two group t-test with a 0.050 two-sided significance level.

As ubiquitin mediated tagging of IKK and Iκβα for proteasomal degradation controls the NFκβ pathway, knowing the minimum number of patients required for immunohistochemistry would help us determine how the levels of proteins tagged by ubiquitin in this pathway differs between the two menstrual cycle phases.

7.2.2 Immunohistochemistry

The phospho NFκβ p65, IKKα, 19S proteasome, IKKβ and phospho Iκβα proteins, were localised according to the method and recommended antibody dilutions in Section 2.2.

7.2.3 Immunohistochemical grading of stained human tissues

A semi-quantitative method, identical to the one for baboons, 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.

7.2.4 Statistical Analysis

Values are expressed as Mean ± SEM, whereby a P-value of < 0.05 was considered statistically significant. This analysis was determined using calculations described in Section 2.1.5.

7.3 Results

7.3.1 IKKβ, Iκβα, NFκβ and IKKα immunostaining

There was no significant difference in the immunostaining for IKKβ (Table 7.1; Figure. 7.1), Iκβα (Table 7.2; Figure. 7.2), NFκβ (Table 7.3, Figure. 7.3) and IKKα (Table 7.4, Figure. 7.4) within the nucleus and cytoplasm of the glands and stroma throughout the menstrual cycle.

Table 7.1. IKK-β protein immunostaining in the endometrium.

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
Secretory	6.95 ± 6.81	2.11 ± 1.54	0.77 ± 0.32	0.42 ± 0.23
P-Value	0.238 (NS)	0.093 (NS)	0.171 (NS)	0.261 (NS)

NS = Not significant.

Table 7.2. Iκβα protein immunostaining in the endometrium.

Phase	Iκβα H-Score			
	Nuclear		*Cytoplasmic*	
	Glands	**Stroma**	**Glands**	**Stroma**
Proliferative	0	0.57 ± 0.56	0.03 ± 0.02	0
Secretory	0	0.02 ± 0.02	0.29 ± 0.21	0.01 ± 0.01
P-Value	N/A	0.162 (NS)	0.099 (NS)	0.159 (NS)

N/A = Not applicable and NS = Not significant.

IKKB

IKBA

Table 7.3. NFκβ protein immunostaining in the endometrium.

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

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

Table 7.4. IKK-α protein immunostaining in the endometrium.

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
Secretory	0	0	0.04 ± 0.04	0.06 ± 0.04
P-Value	0.159 (NS)	0.145 (NS)	0.228 (NS)	0.121 (NS)

NS = Not significant.

NFKB

IKKA

7.3.2 Proteasome immunostaining

A significant increase in proteasome immunostaining within the nucleus of stromal cells was seen at secretory phase compared with the proliferative phase (P = 0.028; Table 7.5; Figure 7.5).

Table 7.5. Proteasome protein immunostaining in the endometrium.

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
Secretory	60.76 ± 40.62	25.40 ± 13.22	3.33 ± 0.93	1.79 ± 0.54
P-Value	0.077 (NS)	0.028 (*)	0.636 (NS)	0.141 (NS)

*P < 0.05 and NS = Not significant.

7.4 Discussion

In this chapter we collected eutopic endometrial tissues from women during the proliferative and secretory phase and examined the levels of downstream proteins within the ubiquitin mediated NFκβ pathway.

The increase in 19S proteasome staining within the nucleus of stromal cells at secretory phase may indicate a greater level of nuclear protein recognition by the 19S protein, as there is a greater requirement for increased protein turnover during this phase. As previously discussed in Section 1.2.2, the secretory phase involves a dramatic change in endometrial cell appearance due to increasing progesterone levels and is where the endometrial lining reaches its maximum thickness. Thus a higher level of 19S protein in this phase could facilitate the turnover of pro and anti-apoptotic proteins such as Bax and Bcl-2 respectively, to control endometrial cell integrity and survival.

Comparable levels of IKKα and IKKβ protein in the nucleus and cytoplasm of proliferative and secretory phase eutopic endometrium suggest a similar kinase activity.

Proteasome

Similar levels of the endogenous inhibitor Iκβα as well as the absence of the NFκβ protein in the nucleus and cytoplasm of eutopic cells, indicate that a different pathway is responsible for endometrial cell differentiation between the proliferative and secretory phase.

In conclusion, the NFκβ pathway does not appear to mediate the endometrial cell differentiation between the proliferative and secretory phase, as supported by the similar IKKα, IKKβ and Iκβα levels observed. The elevated 19S proteasome however, may allow an increased ability to recognize regulatory proteins that control endometrial cell differentiation particularly within the nucleus of stromal cells at secretory phase.