Markers of endometrial function in women with unexplained recurrent pregnancy loss: a comparison between morphologically normal and retarded endometrium

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BACKGROUND: Endometrial defect, usually described as luteal phase defect (LPD), is associated with recurrent miscarriage. Recurrent miscarriage has also been associated with the abnormal expression of various molecules by endometrial cells. The aim of this study was to determine if any of these molecules or cells could be used to distinguish LPD from in-phase endometrium. METHODS: Immunocytochemistry was used to compare endometrial expression of CD45+, CD56+, CD3+ and CD4+ cells, leukaemia inhibitory factor, interleukin-6 and estrogen and progesterone receptors in precisely timed endometrial biopsies obtained between days LH+6 and LH+11 from recurrent miscarriage women with in-phase and retarded endometrium. RESULTS: In all samples there was a positive correlation between the number of CD45+ cells and LH day and a negative correlation between progesterone receptor and LIF expression and LH day. A significantly lower number ($P < 0.05$) of CD56+ cells in peri-implantation endometrium and a decreased mid-cycle estrogen level ($P < 0.05$) was seen in women with LPD compared to in-phase endometrium when single analysis was carried out. However, these differences were not significant after application of the Bonferroni correction for multiple analysis. CONCLUSIONS: The results are in line with previous associations observed between estrogen levels and LPD and suggest that the number of CD56+ cells is different in LPD and in-phase endometrium, although this could be due to delayed endometrial development in women with LPD. Interpretation must be cautious because these differences could have arisen by chance.

Key words: cytokines/endometrium/leukocytes/luteal phase defect/recurrent miscarriage

Introduction

Recurrent miscarriage, defined as a loss of three or more consecutive pregnancies before the 20th week of gestation, may be associated with retarded endometrial development in the peri-implantation period or luteal phase defect (LPD) (Li *et al.*, 2000). Identification of LPD is usually based on morphological study of a precisely timed luteal phase endometrial biopsy according to the classic method of Noyes *et al.* (1950). Although a number of studies have examined endometrial morphology in women with recurrent miscarriage, only two studies (Tulppala *et al.*, 1991; Li, 1998) examined precisely timed endometrial biopsy specimens in women in whom comprehensive investigations into the cause of recurrent miscarriage were conducted. The incidence of LPD in these two studies was reported as 17.4% (Tulppala *et al.*, 1991) and 28% (Li, 1998).

Recent studies have shown differences in expression of a number of endometrial molecules and leukocytes in the peri-implantation period in recurrent miscarriage women, suggesting that these may be useful markers of endometrial function. In particular the measurement of glycodelin A (previously called placental protein PP14) in endometrial flushings has been used successfully to identify endometrial defect (Dalton et al., 1995, 1998). Endometrial leukocyte populations such as stromal CD56+ uterine natural killer cells (uNK, or large granular lymphocytes) (Clifford *et al.*, 1999), and various other stromal leukocyte populations have been examined in recurrent miscarriage (Quenby *et al.*, 1999) and found to be of prognostic value. Differences in endometrial Th1 and Th2 cytokine expression have also been reported to be different in the endometrium of normal subjects and women with recurrent miscarriage (Lim *et al.*, 2000; von Wolff *et al.*, 2000). Endometrial leukaemia inhibitory factor (LIF) is known to be essential for implantation in the mouse (Stewart *et al.*, 1992). A recent study has shown, in contrast to previous observations, that decreased endometrial LIF levels are associated with

increased pregnancy outcome in women undergoing IVF (Ledee-Bataille *et al*., 2002). However, in that particular study, the samples were obtained in the late luteal phase, which is not the optimal time to examine implantation physiology. LIF has been shown to decrease in the late luteal phase (Laird *et al*., 1997; Sharkey, 1998; Robb *et al*., 2002) and it is possible that the higher levels of LIF observed in the late luteal phase in women who failed to conceive is due to delayed appearance of LIF, possibly associated with retarded endometrial development. We and others have shown decreased expression of LIF and interleukin 6 (IL-6), a related cytokine, in some women who suffer recurrent miscarriage (Cork *et al*., 1999; Lim *et al*., 2000; von Wolff *et al*., 2000).

Despite individual reports of differences in expression of these cells and molecules in women with recurrent miscarriage, the relationship between endometrial morphology and their expression has not previously been investigated. The aim of this study was to investigate the relationship between endometrial morphology and a number of potential functional markers in order to determine if any of these biochemical markers could act as markers of endometrial function in women with recurrent miscarriage. Specifically, we wished to compare the expression and location of estrogen receptors (ER), progesterone receptors (PR), leukocyte populations and the cytokine profile including LIF and IL-6 in precisely timed endometrial biopsies taken from women who had suffered recurrent pregnancy loss in the first trimester with normal and retarded endometrial development (LPD). Similar comparisons were also made for mid-cycle plasma estrogen and luteal plasma progesterone levels and luteal phase concentrations of glycodelin A in uterine flushings.

Materials and methods

Human subjects

For the purpose of this study, recurrent miscarriage was defined as a history of three or more consecutive miscarriages in the first trimester, following investigation according to an established protocol (Li, 1998); unexplained recurrent miscarriage was established if all the investigative results were normal. A total of 37 subjects with unexplained recurrent miscarriage were investigated in this study. The study was approved by the local ethics committee and informed consent was obtained from all patients.

Uterine flushing and endometrial biopsy

Daily measurement of LH from mid-follicular phase onwards in either serum or urine was used to identify the LH surge. Flushing of the uterine cavity was carried out as previously described (Dalton *et al*., 1995, 1998) with 2 ml of normal saline solution in the mid to late luteal phase timed precisely according to the LH surge. Endometrial flushings were stored at –20°C until assayed. An endometrial biopsy was obtained with a pipelle sampler (Prodimed, France) immediately after the flushing. The biopsy was immediately placed into formalin fixative, left overnight and then automatically embedded in paraffin wax. Sections were cut at 5 μm, hydrated, stained with haematoxylin and eosin and the histology dated by an experienced gynaecological pathologist according to a previously described method (Noyes *et al*., 1950).

Hormone assay

A mid-cycle blood sample taken at the time of the LH surge was obtained in 24 subjects for estrogen measurement; in addition a mid-luteal phase blood sample for progesterone measurement was obtained in 27 subjects on the day of the endometrial flushing and endometrial biopsy. Serum was immediately separated from cells by centrifugation and stored at –20°C until assayed. Levels of estradiol and progesterone were determined by chemiluminescent immunoassay (Abbott Axsym analyser; Abbott diagnostics). The intra- and inter-assay coefficients of variation were respectively: estradiol, 7.9%, 11.9%; progesterone, 6.8%, 12.4%.

Glycodelin A assay

Glycodelin A in endometrial flushing was measured by radio-immunoassay using a method previously described (Bolton *et al*., 1983). Glycodelin A was iodinated using the chloramine-T method and the resulting tracer was purified through a column of Con A-Sepharose. For the assay, 100 μl of standards or samples was incubated for 24 h at room temperature with 100 μl of 1 ng/ml tracer and 100 μl of antiserum at a dilution sufficient to bind ~45% of the added tracer. A magnetic separating agent (Amerlex-M; Amersham Pharmacia Biotech, UK) was used to separate the antibody-bound glycodelin A from the unbound. Sensitivity of the assay was 3 ng/ml, and the intra- and inter-assay coefficient of variation was <10%.

Immunocytochemistry

Immunocytochemistry was performed on de-waxed, re-hydrated 5 μm tissue sections cut from the same blocks as the sections analysed for routine morphology. The majority of procedures were carried out at room temperature. The exceptions were antigen unmasking, which was carried out in a microwave oven, and incubation with primary antibody, which was performed overnight at +4°C. All sections were quenched in 0.3% hydrogen peroxide in methanol for 20 min prior to antigen unmasking. Unmasking was performed in an 800 W microwave oven; all antigens except CD4 were unmasked in 10 mmol/l citrate buffer pH 6.0. The buffer was heated in the microwave oven until boiling. Slides were then added to the buffer and left at high heat for 2 min. The slides were then incubated for 12 min at medium heat and then left to cool in buffer for 20 min. CD4 was unmasked by heating slides uncovered in 1 litre of 0.1 mol/l EDTA pH 8.0 on maximum heat for 30 min, and then allowing the slides to stand in the heated EDTA for 20 min before washing. All slides were blocked for 1 h in the appropriate blocking buffer (PBS or TBS see below) which contained 250 μl avidin/ml. Slides were then incubated overnight with primary antibody containing 250 μl of biotin/ml. Mouse IgG was substituted for primary antibody in negative controls for mouse anti-human antibodies. In negative controls for rat and goat anti-human antibodies, primary antibody was omitted. The goat anti-human LIF antibody has previously been shown to be absorbed by LIF (Cork *et al*., 1999). Details of primary and secondary antibodies are shown in Table I. Slides were washed twice in either PBS or TBS between each incubation. Binding was visualized by incubation with peroxidase substrate DAB (3,3′-diaminobenzidene tetrahydrochloride; Vector Laboratories, UK). Sections for CD4 were incubated with DAB for 12 min, and all other sections were incubated for 8 min. Slides were then washed in distilled water for 5 min and stained with 10% haematoxylin (Vector) as follows: estrogen receptor (ER) and progesterone receptor (PR) for 20 min, CD45, CD56, CD3, CD4, LIF and IL-6 for 10 min. Slides were differentiated, dehydrated, cleared and mounted in DPX.

Table I. Details of primary and secondary antibodies

Antigen	Primary antibody	Secondary antibody system
ER	Monoclonal mouse@human ER (M7047; Dako, UK) diluted 1:200 in horse serum/PBS[a]	Universal Elite ABC kit (Vector)
PR	Monoclonal mouse@human PR (Novacastra NCL-PGR-312; Vector) diluted 1:100 in horse serum/TBS[b]	Universal Elite ABC kit
CD45	Monoclonal mouse@human CD45 (Dako, M0701) diluted 1:50 in horse serum/TBS	Universal Elite ABC kit
CD56	Monoclonal mouse@human CD56 (MCA 1814; Serotec, UK) diluted 1:100 in horse serum/TBS	Universal Elite ABC kit
CD3	Monoclonal rat@human CD3 (MCA 1477, Serotec) diluted 1:250 in 10% goat serum/TBS	Universal Elite ABC kit secondary antibody substituted + Vector BA-9400 1:100 in 10% goat serum/TBS
CD4	Monoclonal mouse@human CD4 (MCA 1803; Serotec) diluted 1:20 in horse serum/TBS	Universal Elite ABC kit
LIF	Polyclonal goat@human LIF (AF-250-NA; R&D Systems, UK) diluted 1:25 in horse serum/PBS	Universal Elite ABC kit secondary antibody substituted + Vector BA-9500 diluted 1:50 in 2% horse serum/PBS
IL-6	Monoclonal mouse@human IL-6 (MAB-206; R&D Systems) diluted 1:100 in horse serum/PBS	Universal Elite ABC kit

[a]Phosphate-buffered saline, pH 7.5.
[b]Tris-buffered saline, pH 7.6.
ER = estrogen receptor; PR = progesterone receptor; LIF = leukaemia inhibitory factor; IL = interleukin.

Analysis of staining pattern and intensity

Slides were independently scored by two observers. The final result for each parameter was obtained by taking the mean of each individual observer's results. Each individual observer's result was calculated from the analysis of ten fields at ×400 magnification. In the event of large differences between scores, the section was reanalysed by both observers together with a third reviewer. Both observers were blinded with respect to whether the samples were from LPD or in-phase endometrium.

Immunostaining and H-score

ER, PR, LIF and IL-6 staining intensity in tissue sections was graded, and assigned an H-score (Lessey *et al.*, 1996) calculated according to the following equation:

$$H\text{-score} = \sum P_i \, (i + 1)$$

where i = staining intensity (1 = weak, 2 = moderate, 3 = strong) and P_i = percentage of cells staining at each intensity (0–100%). Each parameter was assessed separately in the stroma and in the luminal and glandular epithelium in ten fields at ×400 magnification. In addition the amounts of LIF and IL-6 in the secretions from glandular epithelium were assessed. Inter-observer variation was calculated by taking the mean of the percentage difference between the paired results of each observer. Stromal PR = 0.01%, gland epithelial PR = 0.03%, luminal epithelial PR = 0.02%, stromal ER = 4%, gland epithelial ER = 10%, luminal epithelial ER = 11%, gland epithelial LIF = 0.04%, secreted LIF = 0.02%, gland epithelial IL-6 = 4%, luminal epithelial IL-6 = 7%, and secreted IL-6 = 0.4%.

Gland morphology

The position of the majority of gland epithelium nuclei (basal, middle, or apical), the presence or absence of basal vacuoles, and the distribution of basal LIF staining in sections stained for LIF in the endometrial gland epithelium were recorded.

Leukocyte populations

In sections stained for CD markers, the number of positive cells and the total number of stromal cells in ten high power (×400) microscope fields were counted individually for CD45+, CD56+, CD3+ and CD4+ stained sections. The number of CD+ cells as a percentage of total stromal cell number was then calculated for each tissue section. Inter-observer variation was: CD45+, 0.4%; CD56+, 2.1%; CD3+, 1.7%; and CD3+, 0.9%.

Analysis of data

Biopsies were obtained between days LH+6 and LH+11. The effect of LH day on marker expression was investigated to see if it should be taken into account when comparing in-phase and LPD endometrium; H-scores, cell counts, plasma estrogen and progesterone concentrations and glycodelin A concentrations in uterine flushings were correlated with LH day. The Pearson correlation test was used to investigate the relationship between days past LH surge and each parameter. In a subgroup of women in whom the biopsy was obtained on days LH+7 to LH+9, the H-scores, cell counts, plasma estrogen and progesterone concentrations and uterine glycodelin A values in those with in-phase biopsies were compared to those with retarded biopsies (LPD). Significance of the differences observed between in-phase and LPD biopsies was assessed using the non-parametric Mann–Whitney test. A Bonferroni correction (multiplication by the number of different parameters measured) was also applied to the significance levels obtained to show whether the observed significant differences may have occurred due to multiple analysis. $P < 0.05$ was considered significant. Comparisons were also made between the expression of all the various markers in endometrial biopsies grouped according to whether they were in-phase or LPD. Statistical analysis was computed using SPSS 11.

Photography

Photographs were taken using an Olympus C4040Z digital camera mounted on an Olympus BH2 microscope. Magnification was ×200
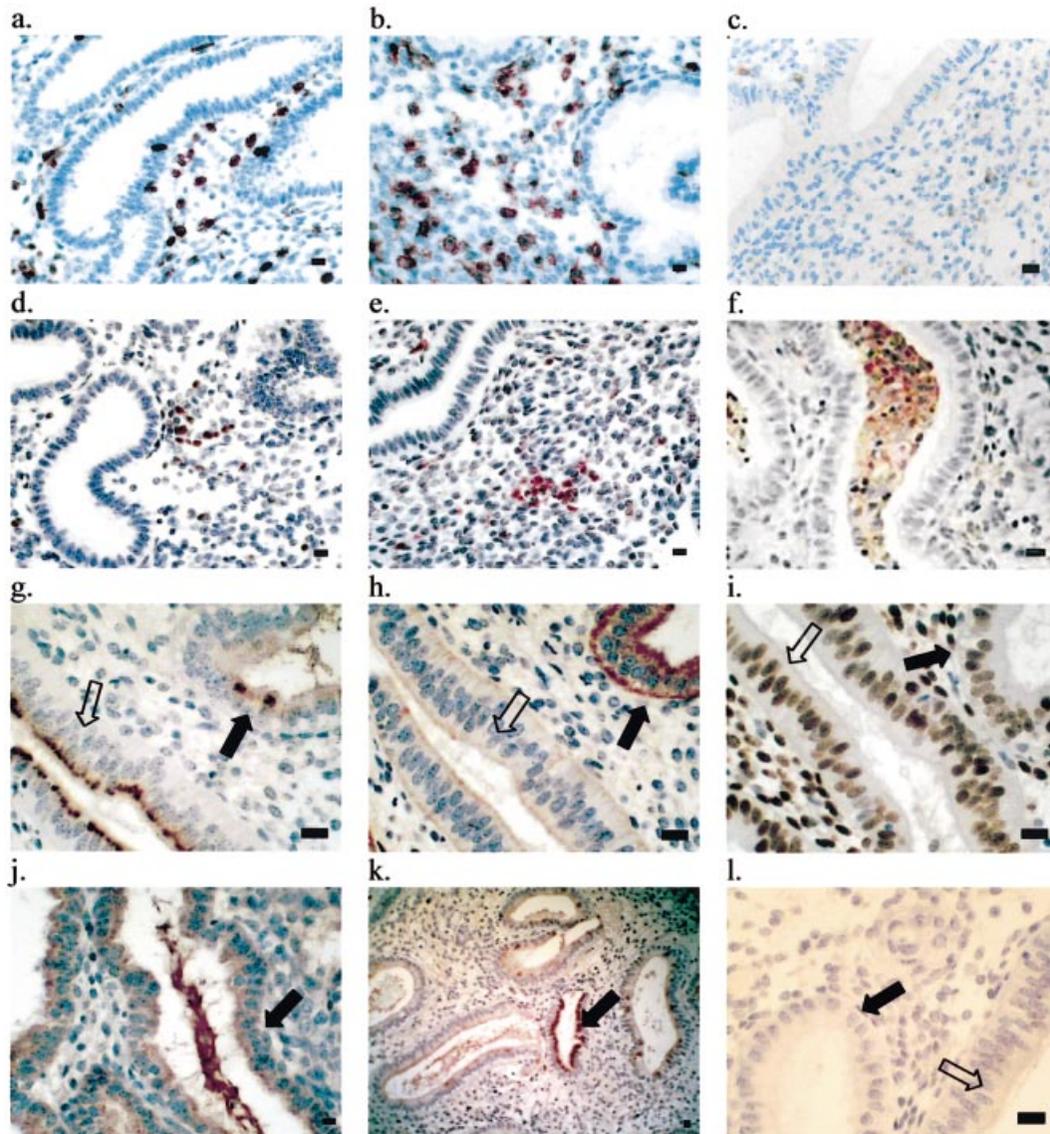
Figure 1. Immunostaining for CD45, CD56, CD3, CD4, interleukin (IL)-6, leukaemia inhhibitory factor (LIF) and progesterone receptor (PR) in the endometrium of women with recurrent miscarriage. (**a**) CD45+ in in-phase endometrium at LH+7, (**b**) high numbers of CD56+ cells in in-phase endometrium at day LH+7, (**c**) low numbers of CD56+ cells in LPD endometrium at day LH+7, (**d**) CD3+ and (**e**) CD4+ cells in in-phase endometrium at day LH+7 and (**f**) CD4+ in gland lumen on day LH+9. (**g**, **h**, **i**) Serial sections stained for IL-6, LIF and PR in in-phase endometrium at day LH+6 (**j**) shows secretion of LIF into gland lumen and (**k**) shows the variation in IL-6 staining in gland epithelium seen within the biopsies. (**l**) IgG1 negative control. Solid arrows: gland epithelium; clear arrows: luminal epithelium. Bar = 10 μm.

with varying degrees of digital zoom. The resulting images were edited using Adobe Photoshop 5.5.

Results

Of the 37 biopsies obtained, one was rejected due to insufficient tissue. Histological dating of the remaining 36 biopsies resulted in 23 being assessed as in-phase (morphologically normal), and 13 assigned a diagnosis of LPD. There were no differences in the age of women in the two groups (LPD mean age = 33 years, range 27–42; in-phase mean age = 33.5 years, range 19–44). Women in the LPD group had suffered significantly more ($P < 0.01$) previous miscarriages than those in the in-phase group (LPD mean number = 5, range 3–8; in-phase mean number = 3, range 3–5).

Pregnancy outcome was assessed in the in-phase group only as the majority of the women diagnosed with LPD underwent treatment in the next cycle. Pregnancy outcomes were available for 21/23 of the in-phase women with recurrent miscarriage, 17/21 women became pregnant, five of these pregnancies resulted in miscarriage and 12 proceeded to a live birth.

Correlation between endometrial markers and chronological dating

Figure 1 shows the immunostaining for CD45, CD56, CD3, CD4, LIF, IL-6 and PR in the endometrial sections. CD45 identified populations of leukocytes in the stroma (Figure 1a). CD56+, CD3+ and CD4+ cells were also mainly present in the stroma (Figure 1b–e), although some CD4+ cells were also seen

Table II. Correlation between LH day and leukocyte populations, steroid receptors, cytokines, and morphological markers in endometrium taken between day LH+6 and LH+11 and plasma progesterone levels in all recurrent miscarriage women ($n = 36$)

Factor measured	Pearson correlation with LH day	*P*-value	*P*-value (Bonferroni corrected)
% CD45+ cells (total leukocytes)	0.65	< 0.001***	< 0.019*
% CD56+ cells (uNK)	0.42	0.011*	0.209
% CD3+ cells (T cells)	0.12	0.473	
% CD4+ cells (helper T cells, macrophages)	0.17	0.330	
H-score			
Stromal PR	0.05	0.756	
Gland PR	−0.72	< 0.001***	< 0.019*
Luminal PR	−0.76	< 0.001***	< 0.019*
Stromal ER	0.19	0.273	
Gland ER	−0.07	0.693	
Luminal ER	−0.24	0.161	
Gland IL-6	−0.28	0.101	
Luminal IL-6	−0.28	0.097	
IL-6 secretion	−0.09	0.597	
Gland LIF	−0.54	< 0.001***	< 0.019*
LIF secretion	0.28	0.095	
Position of gland nuclei	0.49	0.002**	0.038*
% of basal vacuoles	−0.48	0.003**	0.057
Progesterone on day of biopsy ($n = 31$)	−0.50	0.005**	0.095
Glycodelin A in flushing ($n = 17$)	0.53	0.027*	0.513

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
uNK = uterine natural killer cells; ER = estrogen receptor; PR = progesterone receptor; IL = interleukin; LIF = leukaemia inhibitory factor.

within the lumen of the gland in some biopsies (Figure 1f). IL-6 and LIF staining was seen mainly in epithelial cells. IL-6 staining was only present in 22 of the 36 endometrial biopsies. When present, IL-6 staining appeared as small cytoplasmic inclusions, on the apical side of epithelial cells, visible only in a proportion of the glands (Figure 1g, k). When detected in glandular epithelium, IL-6 staining could also be found in luminal epithelium and in some biopsies IL-6 could be seen in the lumen, suggesting secretion from the glands. LIF staining was only seen in glandular epithelium and was absent from luminal epithelium and was seen on both the basal and apical sides of the epithelial cells (Figure 1h). LIF staining was also seen within the lumen of glands, suggesting secretion (Figure 1j).

The relationship between the various endometrial markers measured and days past the LH surge in all biopsy specimens is shown in Table II. The period under study ranged from LH+6 to LH+13 and nine markers showed a correlation with chronological dating during this time. As expected, the traditional criteria used to date the endometrium (position of gland nuclei ($P = 0.002$, non-corrected; $P = 0.038$, after Bonferroni correction) and percentage of epithelial cells with basal vacuoles ($P = 0.003$, non-corrected; $P = 0.057$ after Bonferroni correction) were correlated with LH day. The number of CD45+ ($P < 0.001$, non-corrected; $P < 0.019$ after Bonferroni correction) and CD56+ ($P = 0.011$, non-corrected; $P = 0.209$; after Bonferroni correction) cells in the stroma and the

amount of glycodelin A ($P = 0.027$, non-corrected; $P = 0.513$ after Bonferroni correction) in endometrial flushing on the day of biopsy collection also showed a significant positive correlation with days past the LH surge, although *P*-values were non-significant after Bonferroni correction for CD56+ and glycodelin A. A significant negative correlation with LH day was seen for luteal phase plasma progesterone levels, nuclear PR staining in gland and luminal epithelium (both $P < 0.001$, non-corrected; $P < 0.019$ after Bonferroni correction) and cytoplasmic LIF staining in gland epithelium ($P < 0.001$, non-corrected; $P < 0.019$ after Bonferroni correction). Complete down-regulation of epithelial gland PR was not observed until day LH+11.

Qualitative differences in staining for LIF and IL-6 were also observed as the cycle progressed. LIF staining was initially basal and intense (LH+6–7). As the cycle progressed, staining became increasingly diffuse and could be observed along the apical as well as the basal surface of the cell. The presence of LIF staining in the gland lumen coincided with a reduction of LIF staining in gland cell cytoplasm (Figure 1h, j). IL-6 staining was absent in 13/23 of the in-phase and 9/13 of the LPD biopsies. Down-regulation of PR in gland epithelium did not appear to be a prerequisite for cytokine expression. Figure 1g, h and i shows staining for IL-6, LIF and PR in serial fields from an in-phase biopsy taken at LH+6.

Comparison of endometrial markers between LPD and in-phase endometrium in the peri-implantation period

Table III shows the expression of each marker in LPD and in-phase endometrial biopsies. In order to minimize the effect of differences due to LH day, only biopsies collected at LH+8 ± 1 day (LH+7 to LH+9) were included in this analysis. For the LPD group, this value corresponded to the day the biopsy was collected rather than the day obtained by morphological assessment. Final numbers analysed in each group were 11 in the retarded endometrium group and 20 in the in-phase group. Significantly more basal vacuoles ($P = 0.003$, non-corrected; $P = 0.06$ after Bonferroni correction) and more apical nuclei ($P = 0.014$, non-corrected; $P = 0.28$ after Bonferroni correction) were observed in LPD compared with in-phase endometrium. The number of CD56+ cells in the stroma of LPD biopsies was lower than in in-phase biopsies ($P = 0.049$, non-corrected; $P = 0.98$ after Bonferroni correction) (Figure 1b, c). Peak peripheral blood estrogen levels in the LPD group were lower than in women the in-phase group ($P = 0.041$, non-corrected; $P = 0.82$ after Bonferroni correction). Although gland PR showed a reduction in PR staining in in-phase biopsies compared with LPD biopsies, this result did not reach statistical significance. No differences were seen in the expression of any other markers in retarded and in-phase endometrium.

Correlation of the expression of various markers in in-phase and retarded endometrium

The relationship between expression of the major factors measured within the same biopsy was assessed in LPD and in-phase endometrium, and results are shown in Tables IV and V. Analysis was restricted to biopsies collected on days LH+7, +8

Table III. Endometrial leukocyte populations, steroid receptors, cytokines and morphological markers in endometrial biopsies obtained on days LH+7 to LH+9 of the cycle and hormone levels in women with luteal phase defect (LPD) and in-phase endometrium

	LPD (n = 11)	In-phase (n = 20)	*P*-value	*P*-value (Bonferroni corrected)
% CD45+ (total leukocytes)	20.51 (13.32–27.31)	22.75 (13.48–35.13)	0.183	
% CD56+ (LGL)	7.17 (4.83–15.62)	10.25 (4.14–28.20)	0.049*	0.98
% CD3+ (T cells)	5.25 (1.75–7.02)	3.33 (0–7.96)	0.157	
% CD4+ (T-helper cells)	2.00 (0.50–4.10)	2.50 (0.75–7.20)	0.471	
H-score				
Stromal PR	265 (205–340)	260 (210–320)	0.823	
Gland PR	320 (100–385)	235 (160–365)	0.403	
Luminal PR	270 (60–380)	258 (158–355)	0.359	
Stromal ER	125 (33–245)	130 (0–225)	1.0	
Gland ER	140 (70–330)	145 (20–265)	0.640	
Luminal ER	130 (30–250)	173 (0–250)	0.476	
Gland IL-6	0 (0–253)	0 (0–225)	0.887	
Luminal IL-6	0 (0–340)	15 (0–350)	0.699	
IL-6 secretion	0 (0–100)	0 (0–120)	0.298	
Gland LIF	323 (243–340)	305 (235–360)	0.761	
LIF secretion	0 (0–80)	0 (0–150)	0.123	
Position of gland nuclei	median (apical–basal)	basal (apical–basal)	0.014*	0.28
% of basal vacuoles	30 (0–100)	0 (0–50)	0.003**	0.06
Progesterone on day of biopsy (nmol/l)	39.8 (24.20–72.30)	55.5 (33.10–101.00)	0.106	
	(n = 8)	(n = 19)		
Estradiol at LH peak (pmol/l)	519 (156–1132)	961 (187–1272)	0.041*	0.82
	(n = 9)	(n = 15)		
Glycodelin A in flushing (ng/ml)	38 596 (9840–99 234)	57 848 (5070–157 136)	0.875	
	(n = 6)	(n = 10)		

Values are median (range).
*$P < 0.05$, **$P < 0.01$.
LGL = large granular lymphocytes; ER = estrogen receptor; PR = progesterone receptor; IL = interleukin; LIF = leukaemia inhibitory factor.

and +9. The statistical analysis of these results involved multiple comparisons and therefore some of the statistically significant data may have occurred by chance. Correlations are not shown for position of gland nuclei, luminal epithelial PR and luminal epithelial ER as these results were identical to those for percentage of basal vacuoles, gland PR and gland ER respectively. Otherwise parameters not included in the tables showed no significant correlations with any other parameter studied.

Correlations common to in-phase and LPD recurrent miscarriage endometrium

Correlations that were common to in-phase and retarded endometrium groups are indicated in Tables IV and V. In the stroma, a positive correlation was observed between the number of CD45+ and CD56+ cells, and a negative correlation was observed between the number of CD56+ cells and expression of stromal PR. Expression of gland PR showed a positive correlation with the presence of basal vacuoles, and expression of gland ER correlated with expression of stromal ER.

Correlations specific to in-phase recurrent miscarriage endometrium

In in-phase endometrium, positive correlation was observed between serum progesterone levels at the time of biopsy and

collection and expression of gland ER. Mid-cycle serum estrogen levels correlated negatively with staining for gland ER and positively with the number of CD56+ cells in the stroma. In in-phase, endometrium levels of LIF, IL-6 and glycodelin did not show correlation with any other factor measured.

Correlations specific to retarded recurrent miscarriage endometrium

In retarded endometrium, the level of glycodelin in endometrial flushing correlated negatively with expression of both PR and LIF in epithelial gland cells. Epithelial gland IL-6 expression showed negative correlation with the number of CD45+ cells in the stroma. In retarded endometrium, no correlations were seen between plasma estrogen or progesterone levels and any other factor.

Correlation of markers with pregnancy outcome

Among the 17 subjects with in-phase endometrium who subsequently conceived, there was no detectable difference in any of the markers between those who subsequently miscarried (n = 5) and those who subsequently had a live birth (n = 12). In women with LPD biopsies, a positive relationship was observed between the number of previous miscarriages and the numbers of CD45+ ($P < 0.05$) and CD56+ ($P < 0.05$) cells in

Table IV. Correlation between the major factors measured in in-phase endometrium

	CD45	CD56	CD3	CD4	Stromal PR	Gland PR	Stromal ER	Gland ER	Basal vacuoles	Progesterone	Estradiol
CD45	_	r = 0.47*a	NS	NS	NS	r = −0.65**	NS	NS	NS	NS	NS
CD56	r = 0.47*a	_	NS	r = −0.50*	r = −0.46*a	r = −0.45*	NS	NS	NS	NS	r = 0.53*
CD3	NS	NS	_	NS	NS	NS	r = 0.52*	NS	NS	NS	NS
CD4	NS	r = −0.50*	NS	_	NS	NS	r = 0.60**	NS	r = 0.53*	NS	NS
Stromal PR	NS	r = −0.46*a	NS	NS	_	r = 0.47*	NS	NS	NS	NS	NS
Gland PR	r = −0.65**	r = −0.45*	NS	NS	r = 0.47*	_	r = 0.61**	NS	r = 0.45*a	NS	NS
Stromal ER	NS	NS	r = 0.52*	r = 0.60**	NS	r = 0.61**	_	r = 0.55*a	NS	NS	NS
Gland ER	NS	NS	NS	NS	NS	NS	r = 0.55*a	_	NS	r = 0.47*	r = −0.56*
Basal vacuoles	NS	NS	NS	r = 0.53*	NS	r = 0.45*a	NS	NS	_	NS	NS
Progesterone	NS	NS	NS	NS	NS	NS	r = 0.47*	NS	NS	_	NS
Estradiol	NS	r = 0.53*	NS	NS	NS	NS	NS	r = −0.56*	NS	NS	_

[a]Correlations that were observed in both in phase and luteal phase defect biopsies.
*$P < 0.05$, **$P < 0.01$.
PR = progesterone receptor; ER = estrogen receptor; NS = non-significant.

the stroma, but this was not seen in women with in-phase endometrium.

Discussion

Abnormal endometrial development (LPD) is a contributory factor to recurrent miscarriage; fetal or embryo abnormality is another important cause. The aim of this study was to investigate whether endometrial expression of specific cellular and molecular markers differ in women with in-phase and LPD endometrium. Endometrial expression of these markers will be menstrual cycle dependent, and therefore timing of the biopsy is crucial. In this study, timing of the biopsy was accurately determined from the day of the LH surge. The endometrial biopsies used in this study were from archived material, which meant that only certain factors could be measured. We chose to study steroid receptors, leukocyte populations and the cytokines LIF and IL-6, as the pattern of expression of these markers in the endometrium throughout the normal menstrual cycle has been well documented (Lessey *et al.*, 1988; Press *et al.*, 1988; Bulmer *et al.*, 1991; Laird *et al.*, 1997; von Wolff *et al.*, 2002). One of the limitations of the present study is that there is no comparison with normal fertile women. Precisely timed endometrial tissue from normal fertile women is in extremely short supply and therefore, as previous studies have already shown abnormal expression of the markers studied in women with recurrent miscarriage (Lessey *et al.*, 1996; Lachapelle *et al.*, 1996; Clifford *et al.*, 1999; Cork *et al.*, 1999; Quenby *et al.*, 1999; von Wolff *et al.*, 2000), the results were not repeated in this study. In addition, we wished to directly address the question about differences in LPD and in-phase endometrium; inclusion of a normal fertile group, although desirable, was not essential for this purpose.

In this study, as multiple comparisons were made between the in-phase and LPD groups, it increases the likelihood that the observed differences between groups occur merely due to chance. We have therefore also applied the Bonferroni correction to the *P*-values which showed significant differences between groups. However, it is possible that the rigorous adherence to such a stringent significance level would reduce the sensitivity of the study to detect a marker that is of potential importance and therefore we have also shown the non-corrected significance levels.

Correlation of factors with cycle day
Steroid receptors
The rising levels of progesterone during the luteal phase of the cycle induces down regulation of PR epithelial cell expression and this is indicated in this study by the negative correlation between LH day and PR epithelial cell expression. Previous studies (Lessey *et al.*, 1988), including those published from our laboratory (Li *et al.*, 2002), have shown very little epithelial PR expression after day LH+8, which is in contrast to the results of the present study in which epithelial PR staining was seen up to day LH+11 with maximum down-regulation occurring between days LH+9 and LH+11. This is likely to be due to the use of a new PR antibody (Novacastra NCL-PGR-312 instead of Novacastra NCL-PGR clone 1 A6), which is specific for an N-terminal sequence in the A form of PR.

Leukocyte populations
The leukocyte populations within the endometrium consist mainly of T cells, macrophages and uNK cells (Bulmer *et al.*, 1991; Johnson *et al.*, 1999). The majority of these cells express CD56 and CD38, but not the classical T (CD3) or NK (CD16)

Table V. Correlation between the major factors measured in luteal phase defect (LPD) endometrium

	CD45	CD56	CD3	CD4	Stromal PR	Gland PR	Stromal ER	Gland ER	IL-6	LIF	Basal vacuoles	Glycodelin
CD45	_	$r = 0.78$**a	$r = 0.71$*	NS	NS	NS	NS	NS	$r = -0.83$**	NS	NS	NS
CD56	$r = 0.78$**a	_	NS	NS	$r = 0.74$**a	NS	$r = 0.61$*	NS	NS	NS	NS	NS
CD3	$r = 0.71$*	NS	_	NS	NS	NS	NS	NS	NS	NS	$r = 0.7$*	NS
CD4	NS	NS	NS	_	NS	NS	NS	$r = 0.62$*	NS	NS	NS	NS
Stromal PR	NS	$r = 0.74$**a	NS	NS	_	NS	$r = 0.63$*	NS	NS	NS	NS	NS
Gland PR	NS	NS	NS	NS	NS	_	NS	NS	NS	NS	$r = 0.72$*a	$r = -0.87$*
Stromal ER	NS	0.61*	NS	NS	0.63*	NS	_	$r = 0.79$**a	NS	NS	NS	NS
Gland ER	NS	NS	NS	$r = 0.62$*	NS	NS	$r = 0.79$**a	_	NS	NS	NS	NS
IL-6	$r = -0.83$**	NS	NS	NS	NS	NS	NS	NS	_	NS	NS	NS
LIF	NS	NS	NS	NS	NS	NS	NS	NS	NS	_	NS	$r = -0.93$**
Basal vacuoles	NS	NS	$r = -0.70$*	NS	NS	$r = 0.72$*a	NS	NS	NS	NS	_	NS
Glycodelin	NS	NS	NS	NS	NS	$r = -0.88$*	NS	NS	NS	$r = -0.93$**	NS	_

aCorrelations that were observed in both in phase and LPD biopsies.
**$P < 0.01$ ***$P < 0.001$.
PR = progesterone receptor; ER = estrogen receptor; IL = interleukin; LIF = leukaemia inhibitory factor; NS = non-significant.

cell markers (Bulmer *et al*., 1991). The results of this study show a positive correlation between LH day and the total number of leukocytes, particularly CD45+ and also CD56+ cells. In contrast, there was no correlation between CD3+ and LH day. These results concur with previous studies that have shown a gradual increase in leukocyte numbers in the endometrium during the secretory phase of the menstrual cycle, mainly due to increased CD56+ cells (King *et al*., 1989; Bulmer *et al*., 1991).

Cytokines
Alterations in endometrial LIF and IL-6 expression throughout the menstrual cycle is well documented with maximum expression during the mid–late secretory phase of the cycle (Laird *et al*., 1997; Sharkey *et al*., 1998; von Wolff *et al*., 2002). The negative correlation between LH day and epithelial cell LIF expression seen in this study suggests that LIF expression decreases from days LH+6 to LH+11 of the cycle. This would agree with other studies that have shown that LIF expression is maximal at the time of implantation (Laird *et al*., 1997; Sharkey *et al*., 1999). In contrast to LIF, no correlation between IL-6 expression and LH day was seen. As suggested by others (Sherwin *et al*., 2002; von Wolff *et al*., 2002), endometrial IL-6 expression was extremely variable between women.

Expression of factors in in-phase and LPD endometrium
Because of the differences in expression of some of the endometrial components at different days in the cycle, comparisons between in-phase and LPD endometrium were restricted to tissue obtained on days LH+7, LH+8 and LH+9. LPD is defined as morphological dating >2 days behind the LH date. As expected, a significant difference was seen in the classical markers of endometrial morphology (positioning of gland nuclei and percentage basal vacuoles) between in-phase and LPD endometrium.

Steroid receptors
A previous study has shown increased epithelial cell expression of PR in women with recurrent miscarriage and LPD (Lessey

et al., 1996) consistent with retarded endometrial development. In this study, there were no differences in either PR or ER expression between in-phase and LPD endometrium from recurrent miscarriage women. This may be due to the time in the menstrual cycle that the biopsies were taken. The down-regulation of PR in this study appeared to occur between days LH+9 and LH+11 and therefore study of endometrium at days LH+7 to LH+9 may not detect differences in PR expression.

Cytokines
Previous studies have shown decreased expression of endo-metrial LIF and IL-6 in women with recurrent miscarriage (Cork *et al*., 1999; Lim *et al*., 2000; von Wolff *et al*., 2000). In this study, there were no differences in endometrial LIF and IL-6 expression between in-phase and LPD biopsies, suggesting that the decreased expression seen in previous studies is independent of LPD.

Leukocyte populations
Previous studies have suggested increased endometrial expres-sion of CD56+ cells in women with recurrent miscarriage (Clifford *et al*., 1999; Quenby *et al*., 1999). In this study, there were more CD56+ cells present in in-phase endometrium compared with retarded endometrium although the difference was not significant after Bonferroni correction. The increased number of CD56+ cells seen in women with recurrent miscarriage may be independent of the endometrial defect associated with LPD. More recent studies have suggested the presence of subsets of CD56+ cells in the endometrium. In particular, an increased expression of CD56+CD16+ cells, but not total CD56+ cell number, in the endometrium of recurrent miscarriage has been reported (Lachapelle *et al*., 1996). The limited amount of tissue available for this study meant that the presence of endometrial CD16+ cells was not investigated and therefore we cannot assess which population of endometrial CD56+ cells was decreased in women with LPD.

Studies have suggested that >50% of the products of conception from women with recurrent miscarriage have an abnormal karyotype (Stern *et al*., 1996) and that populations of both peripheral blood and decidual NK cells are different in

recurrent miscarriage women with chromosomally normal and abnormal fetuses (Yamamoto *et al.*, 1999; Yamada *et al.*, 2001). Yamamoto et al. showed that the percentages of both CD56+CD16+ and CD56+CD16− decidual leukocytes were decreased in women with missed abortions of fetuses with normal chromosomes compared to those with missed abortions of fetuses with abnormal chromosomes. However, the CD56+CD16+/CD56+CD16− ratio was higher in decidua of women with karyotypically normal fetuses (0.117) compared to women with karyotypically abnormal fetuses (0.095). A recent study has also suggested increased CD16+ cells in early pregnancy decidua of women with recurrent miscarriage (Emmer *et al.*, 2002). In contrast, Yamada et al. showed increased cytolytic activity in peripheral blood NK cells from recurrent miscarriage women with chromosomally normal fetuses compared to those with chromosomally abnormal fetuses. Taken together, these studies suggest the importance of studying subpopulations of CD56+ cells in recurrent miscarriage and further work on their expression in the endometrium of women with recurrent miscarriage should be carried out.

No differences were seen in expression of CD3+ and CD4+ cells between in-phase and LPD endometrium, again suggesting that the previously reported differences seen in endometrial CD4+ cells in women with recurrent miscarriage (Lachapelle *et al.*, 1996; Quenby *et al.*, 1999) are independent of LPD. No attempt was made to look for differences in other subpopulations of CD3+ cells. Although there appear to be no differences in total decidual or endometrial T cell numbers in women with recurrent miscarriage (Lachapelle *et al.*, 1996; Quenby *et al.*, 1999; Quack *et al.*, 2001), differences in CD25+ (T cell activation marker) expression in decidua of recurrent miscarriage and normal fertile women has been reported (Quack *et al.*, 2001) and would therefore justify further study in the endometrium.

Steroid hormone levels

There was no difference in luteal phase progesterone levels in women with in-phase and retarded endometrium, which supports our previous observation that most cases of LPD are associated with normal progesterone levels (Li *et al.*, 2000) and that treatment with high doses of progesterone in the luteal phase does not advance the endometrium in either normal or artificial cycles (Li *et al.*, 1991). In contrast, LPD was associated with low mid-cycle plasma estrogen levels, which is consistent with our previous observation that follicular phase estrogen priming is important in luteal phase endometrial development (Li *et al.*, 1994). Low estrogen levels might also indicate low oocyte quality and result in a corpus luteum that is not functioning normally although secreting normal amounts of progesterone. If decreased corpus luteal function is the cause of retarded endometrial development, rather than (or as well as) low estrogen levels, the results from this study suggest that retardation is not due directly to changes in progesterone levels. It may be that another factor produced by the corpus luteum is important, or that progesterone's effect on the endometrium is mediated by other factors which are deficient in women with LPD.

Correlation with pregnancy outcome

There was no correlation between levels of expression of any of the endometrial markers and pregnancy outcome. Although there are no studies on the predictive value of endometrial IL-6, LIF, or steroid receptor expression on pregnancy outcome, a previous study showed that women who subsequently miscarried had more endometrial CD56+ cells than those who subsequently had a live birth (Quenby *et al.*, 1999). In our study, the number of women who became pregnant ($n = 17$) was probably too small to detect any significant correlation.

In conclusion, this study has investigated the expression of postulated markers of endometrial function in peri-implantation endometrium from women with recurrent miscarriage. It has shown that expression of some of these markers changes considerably during this period and therefore, when making comparisons between individuals, timing of the biopsy is important. There were no differences in endometrial expression of CD45+, CD4+, CD3+ cells, ER and PR and LIF and IL-6 between in-phase and retarded endometrium, suggesting that these are not suitable endometrial biochemical markers of LPD. A decreased expression of endometrial CD56+ cells and follicular phase estradiol levels was seen in women with endometrial defect, although these differences were not significant when corrected for multiple analysis and need to be tested for reproducibility in other studies.

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