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Endometrial factors in recurrent miscarriage

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Recurrent pregnancy loss may be a consequence of an abnormal embryonic karyotype, or maternal factors affecting the endometrium resulting in defective implantation. In order to study the endometrial factors responsible for recurrent pregnancy loss, endometrial biopsy samples should be precisely timed according to the LH surge, and the investigation should be carried out in a non-conception cycle, prior to the next pregnancy. The various methods of studying the endometrium including morphological studies, morphometry, immunohistrochemistry, measurement of endometrial protein in plasma and uterine flushings, cytokine expression in endometrial cells, leukocyte populations in the endometrium and ultrasonographic and hysteroscopic studies, were reviewed. The clinical relevance of the observed abnormality depends on whether or not the abnormality is persistent in subsequent cycles, and if the observed abnormality is of significant prognostic value. Very little is known about the treatment of endometrial defect associated with recurrent pregnancy loss, but preliminary data suggest that the use of HMG may be of benefit.

Keywords: endometrium/endometrial investigations/implantation/recurrent miscarriage

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Introduction

Why study the endometrium in recurrent miscarriage? For pregnancy to be successful, the embryo must be able to implant into the endometrium, from which the embryo derives nutrition and support for continuing growth. The process of implantation is now understood to require a series of finely orchestrated events to take place between the embryo and the endometrium. Approximately 50% of miscarriages in women with a history of recurrent loss are associated with an abnormal embryonic karyotype. The remaining 50% may therefore be due to factors that cause alteration of one or more of the many other components that are essential for successful embryo/endometrial dialogue, implantation and continuing pregnancy. A recent study (Ogasawara *et al.*, 2000) has shown that the frequency of normal embryonic karyotypes significantly increases with the number of previous miscarriages. This observation indirectly suggests that as the number of miscarriages increase, maternal factors involved in embryo/endometrial dialogue may become increasingly responsible for pregnancy failure.

Timing of the investigation

The strategy for studying the endometrium in reproductive failure is very different to the investigation of endometrium in women with abnormal bleeding or who are suspected of having neoplastic disease. If the primary concern is neoplastic disease of the endometrium (which does not undergo significant changes in the menstrual cycle), the timing of the investigation is relatively unimportant. However, if the main purpose of the investigation is to establish possible causes for reproductive failure, the timing of the investigation becomes crucially important and should, in general, be carried out at the peri-implantation period, that is in the mid-luteal phase. In addition, as endometrial structure and function changes rapidly throughout the menstrual cycle, it is important to know exactly at what stage of the menstrual cycle the investigation was carried out. In the past, the onset of the menstruation was used as the reference point, but it has now been widely acknowledged that a much more precise reference point for timing the investigations is either the LH surge as determined by daily LH monitoring on plasma or urine samples (Li *et al.*, 1987), or follicle rupture (ovulation) as detected by daily follicle

scanning (Shoupe *et al.*, 1989). To investigate the endometrium in recurrent pregnancy loss without knowledge of the precise timing of the collection of the sample (chronological dating) is rather meaningless and a waste of time.

Finally, while it is possible to investigate the endometrium in a conception cycle, there are a number of limitations with this approach. There are important ethical considerations, making it difficult to conduct invasive tests such as endometrial biopsy, especially in control subjects in whom the pregnancy is progressing normally. Even if significant changes are detected in a failing pregnancy, it is difficult to be sure whether the changes represent the cause or effect of the failure. Hence investigations of the endometrium are better carried out in a non-conception cycle, prior to the next pregnancy.

Methods of investigation

For many years, the study of the human endometrium, both in physiological and pathological conditions, has been based primarily on histological examination of biopsied specimens. Nowadays, many different methods are available to investigate the endometrium in women with recurrent miscarriage (RM):

1. Morphological study using conventional histological criteria (Noyes *et al.*, 1950).
2. Quantitative histology/morphometry.
3. Immunohistochemical study.
4. Measurement of endometrial protein and other constituents in uterine flushings.
5. Measurement of endometrial protein in plasma.
6. Cytokine expression in endometrial cells.
7. Leukocyte population in endometrial and decidual tissue.
8. Others, such as ultrasonographic study and hysteroscopic examination of the endometrial cavity.

Morphological study

Histological examination of precisely timed endometrial biopsy in the luteal phase is the classic method used to evaluate the normality of endometrial development. The traditional dating criteria described (Noyes *et al.*, 1950) have been widely used. A number of studies have examined abnormal endometrial morphology in women with recurrent miscarriage (RM); unfortunately, many of them were of limited value because they either did not employ strict criteria for the diagnosis of RM and included women with two miscarriages only (Tho *et al.*, 1979; Balasch and Vanrell, 1986; Davidson *et al*., 1987), the number of miscarriages was not clearly stated (Daya *et al.*, 1988), or the patients did not have comprehensive investigations for the various underlying causes and the endometrial biopsies were not accurately timed by the LH surge (Grant *et al.*, 1959; Llusia, 1962). There were only two morphological studies which employed a precise method (LH surge) to time the endometrial biopsy and had comprehensive investigations for the RM; the incidence of endometrial defect in these two studies was reported as 17.4% (Tulppala *et al.*, 1991) and 28% (Li, 1998).

Morphometric analysis

Morphometric analysis provides a quantitative analysis of the endometrium. The application of morphometric techniques, for example cell count, point counting and calculation of volume fraction, to the study of the human endometrium has provided valuable information on the response of the different components of the endometrium to peripheral steroid hormones (Johannisson *et al.*, 1982, 1987; Li *et al.*, 1988). Whereas the traditional criteria (Noyes *et al.*, 1950) provides an overall assessment of endometrial development, morphometric analysis permits an in-depth study of the changes involving different components of the endometrium. Morphometric analysis was employed to examine endometrial biopsy specimens from women with RM (Serle *et al*., 1994), and it was noted in a subgroup of women with RM, that there was a significant reduction in the volume fraction of endometrium occupied by gland, the volume fraction of gland occupied by lumen, and the number of subnuclear vacuoles per 100 gland cells. Others (Saleh *et al*., 1995) examined the luminal epithelium and noted that the cells in women with RM were significantly shorter than those of control fertile subjects.

Immunohistochemistry

In recent years, the application of immunohistochemical techniques has permitted the study of a large number of specific proteins in the endometrium. There have been two studies which employed immunohistochemical techniques to the study of expression of endometrial proteins in women with RM. It was found (Serle *et al.*, 1994) that the expression of four mucin-related secretory epitopes was reduced in women with RM. Moreover, these authors found that there was a greater reduction associated with morphological retardation. Others (Hey *et al*., 1995) found that the expression of a polymorphic epithelial mucin MUCI, a cell-surface and secretory product of endometrial epithelium, was also reduced in women with RM. There are, of course, many other protein markers, including pregnancy-associated endometrial α_1 and α_2 globulins (Bell *et al.*, 1985), placental protein 14 (Julkunen *et al.*, 1990), 24K protein (Ciocca *et al.*, 1983), CA125 (Kabawat *et al.*, 1983), prolactin (Kauma and Shapiro, 1986), a keratin sulphate 5D4 (Hoadley *et al.*, 1990), laminin (Aplin *et al.*, 1988), a sialo-glycoprotein D9B1 epitope (Seif *et al.*, 1989), 17-beta-hydroxysteroid dehydrogenase (Maentausta *et al.*, 1990) and a stromal protein desmin (Halperin *et al.*, 1991), as well as the use of lectin binding to study glycoproteins (Klentzeris *et al.*, 1991). So far, the immunocytochemical analysis of these markers has not been used to study the endometrium of women with recurrent pregnancy loss.

Uterine flushing

The secretory activity of endometrial glands may be assessed by measuring the concentration of specific proteins in the uterine flushing. The techniques of uterine flushing and the concentration of an endometrial secretory protein, placental protein 14 (PP14) or glycodelin A, in uterine flushing throughout the menstrual cycle of normal fertile subjects has been described (Li *et al.*, 1993a,b). The concentration of PP14 and MUCI were both reduced in women with RM (Dalton *et al.*, 1995; Hey *et al.*, 1995; Aplin *et al.*, 1996) when compared with controls; however, the concentration of CA125 in uterine flushings was similar to those of fertile controls (Dalton *et al.*, 1995).

Endometrial protein in plasma

The endometrial protein PP14 may also be measured in plasma samples. One study found that plasma PP14 concentrations in

women with RM were lower than in controls (Tulppala *et al*., 1995), but another showed no difference (Dalton *et al.*, 1998). Whilst there was a significant correlation between plasma and uterine PP14 concentrations in women with RM (Dalton *et al.*, 1998), measurements of PP14 in uterine flushings appeared to be more discriminatory than those in plasma samples.

Endometrial cytokines expression

In murine models, pregnancy rejection is mediated by T-helper-1 (TH-1) cytokines, which include interleukin (IL)-2 and interferon (IFN)-γ; whereas successful implantation and pregnancy depends on the presence of T-helper-2 (TH-2) cytokines, such as IL-4, IL-6 and IL-10, which promote trophoblastic growth (Wegmann *et al*., 1993).

The endometrial cytokine expression in the peri-implantation period was examined using RT–PCR and enzyme-linked immunosorbent assay (ELISA) in 25 women with RM, and results were compared with those in 10 fertile control subjects (Lim *et al.*, 2000). It was found that women with RM had higher levels of TH-1 cytokines [IFN-γ, IL-2, IL-12, tumour necrosis factor (TNF)-β] and lower levels of TH-2 cytokines (e.g. IL-6) than control subjects in both the endometrium and blood. There was no apparent correlation between cytokine expression and various serum hormone levels (FSH, LH, estradiol, progesterone and testosterone) of samples obtained at the time of collection of endometrial samples.

In another study, immunohistochemistry was used to study the expression of two cytokines, leukaemia inhibitory factor (LIF) and IL-6 in 18 women with RM, with results being compared to those in fertile controls (Cork *et al*., 1999). The expression of LIF and IL-6 was reduced in 31% and 11% respectively of women with RM. This reduced expression of endometrial IL-6 in RM women has recently been confirmed by others using RNase protection assays (Von Wolff *et al.*, 2000).

Endometrial leukocytes

The cytokine profile of the endometrium may be closely related to the leukocyte population in the endometrial stroma. At about the time of implantation, approximately 20% of endometrial stroma cells are leukocytes, of which the majority are large granular lymphocytes (LGL) (Bulmer *et al.*, 1987). The cytoplasmic granules of LGL contain cytolytic molecules (King *et al.*, 1993) which may play an important role in limiting trophoblast invasion into the decidua (King and Loke, 1991).

Another group (Lachapelle *et al*., 1996) studied the leukocytes in endometrial biopsy from 20 women with RM and 15 fertile control subjects using two-colour flow cytometric analysis. These authors found that the percentage of endometrial CD8+ T lymphocytes was significantly reduced in women with RM, whereas the proportion of CD20+ T lymphocytes was strikingly increased. Whilst the proportion of natural killer (NK) cells was similar in women with RM and fertile controls, the CD16+ $CD56^{dim}$ subset was increased but CD16– $CD56^{bright}$ was reduced in women with RM. Furthermore, it was found that women with RM who had normal CD8 and CD20 expression subsequently underwent successful pregnancies, while those with abnormal expression continued to have miscarriages. Similarly, the proportion of CD16– $CD56^{bright}$ to CD16+/$CD56^{dim}$ NK cells also had prognostic value.

The leukocyte population was also examined in the mid-luteal phase of the endometrium of 22 women suffering from unexplained RM (three or more), with results being compared with those of nine control subjects (Quenby *et al*., 1999). All the biopsies were timed from the last menstrual period. These authors found similar numbers of CD 3+ and CD 8+ cells in both groups. However, the positive staining of at least one of CD 4+, CD 14+, CD 16+ and CD 56+ and of MHC class II+ cells in the RM group was significantly higher than in the control group. Moreover, it was found that within the group of women with RM, those who had a further subsequent miscarriage after the endometrial study had greater staining of CD 56+ cells than those whose subsequent pregnancy resulted in a live birth.

The CD 56+ cells in the endometrium of 29 women with RM were examined and compared with those of 10 control subjects (Clifford *et al*., 1999). All the endometrial biopsies were taken in the luteal phase, from days LH +7 to LH +10. The authors employed more refined, quantitative methods of evaluation and measured the number of CD 56+ cells per 10 high-power fields. The earlier findings (Quenby *et al.*, 1999) were confirmed in that there were significantly more endometrial CD 56+ cells in women with RM than in the control group. Moreover, no correlation was found between the number of CD 56+ cells and the maternal age, the number of previous miscarriages, a past history of a live birth and the time since last miscarriage. Interestingly, it was found that among women with RM, those ($n = 6$) with at least one late loss (>13 weeks) did not appear to have an increase in CD 56+ cells compared with the control group. The last two studies suggest that RM is associated with an alteration in the endometrial leukocyte population, especially an increase in CD 56+ cells.

Others (Hill *et al*., 1995) examined the leukocyte population in the decidual tissue of three groups of subjects: (i) those with first spontaneous miscarriage ($n = 8$); (ii) those with unexplained spontaneous RM ($n = 20$); and (iii) those having elective termination of pregnancy ($n = 20$). It was found that the pattern of CD56+ staining was not significantly different between groups, except for a paucity of CD56+ leukocytes in those with first spontaneous miscarriage. Current evidence indicates that endometrial leukocytes play an important role in the process of implantation by regulating the extent of trophoblastic invasion (King *et al.*, 1998).

It was also found (Lea *et al*., 1997) that the co-expression of bcl-2 (an inhibitor of aptoptosis, i.e. programmed cell death) in CD56+ cells is enhanced during pregnancy, suggesting that these cells are selected for survival and play an important role in implantation and early pregnancy. Bcl-2 immunoreactivity in syncytiotrophoblast tissues obtained from surgically terminated normal pregnancy ($n = 22$) was higher than those from either sporadic ($n = 16$) or recurrent ($n = 22$) miscarriages. However, it is still unclear if this difference was a cause or effect of the miscarriage.

In studying leukocyte activation in the decidua of RM, it was found that significantly more activated leukocytes were present in the decidua of women with unexplained RM who had a normal male karyotype, compared with women with a trisomy miscarriage or normal pregnancies following elective termination procedures (Quack *et al*., 2001). In addition, the number of cells comprising the major leukocyte subpopulation, CD56+ NK cells, appeared to be reduced in the decidua of women with unexplained

RM compared with decidua from women having elective termination. In contrast, it was interesting to note that there were fewer CD56+ cells in the endometrium of women with unexplained infertility, compared with fertile controls (Klentzeris *et al*., 1994).

On the basis of the various morphological, biochemical, cytokine and immunohistochemical studies presented above, there seems little doubt that an endometrial defect is associated with RM. However, whether the relationship is a causal or casual one remains to be determined.

Other methods of investigation

Imaging techniques of the endometrium and uterus including pelvic ultrasonography and magnetic resonance imaging (MRI), as well as direct visualization of the endometrial cavity by hysteroscopy are useful in the diagnosis of various structural anomalies associated with recurrent pregnancy loss such as uterine septum, intrauterine synechiae and uterine fibroids (see Uterine pathology).

Pathophysiology of endometrial abnormalities

Endometrial abnormality detected at about the time of implantation may be a result of endocrinological factors, or secondary to local, uterine pathology.

Endocrinological factors

Endometrial development and differentiation is highly dependent on steroid hormones. During the follicular phase, the developing follicle produces estrogen which acts on the endometrium leading to proliferation and the production of progesterone receptors (PR). After ovulation, the corpus luteum produces progesterone which then interacts with PR in the endometrium to produce the characteristic sequence of development. Thus, abnormal endometrial development could be a result of an aberrant follicular phase resulting in inadequate priming of the endometrium, subnormal progesterone production by the corpus luteum, or an abnormal response of the endometrium to progesterone.

Aberrant follicular phase

It is possible that aberrant follicle development (of which polycystic ovaries are an example) leads to an increased risk of miscarriage. This may occur via two mechanisms: (i) the production of an oocyte of suboptimal quality; and (ii) by subnormal estrogen production and inadequate priming of the endometrium, leading to delayed development in the luteal phase. It has been shown that women with retarded endometrial development in the luteal phase had significantly lower FSH levels in the follicular phase of the same cycle (Cook *et al.*, 1983). In a recent study (Li *et al.*, 2000), the hormone profile in women with RM was examined, including: (i) a blood sample in the early follicular phase to measure FSH, LH, testosterone, androstenedione, sex hormone-binding globulin (SHBG); and (ii) daily blood samples from the mid-follicular phase onwards for LH and estradiol measurement until the LH surge is identified. An endometrial biopsy was obtained later in the mid-luteal phase. Among the 122 women with RM who were studied, 89 had normal endometrial development, whereas 33 had delayed development. The hormonal data obtained from these groups of women were compared (Li *et al.*, 2000), and showed that the parameters in the follicular phase did not differ significantly between the two groups. However, it will be of interest to conduct a prospective study to examine the follicular phase in detail including ovarian follicle tracking along with Doppler blood flow (as well as serial endocrinological measurements) in women with RM, and to correlate the results to observations in the luteal phase, including endometrial morphology and immunohistochemistry.

Table I. Correlation of endometrial dating with plasma progesterone results in the mid-luteal phase in women with recurrent pregnancy loss

Plasma progesterone (nmol/l)	Endometrial dating	
	Normal	Retarded
⩾30	55	16
<30	7	8

Normal versus retarded $\chi^2 = 5.8$, $P < 0.02$.

Subnormal progesterone production

Subnormal progesterone production by the corpus luteum may result in inadequate stimulation and development of the endometrium. In the above-mentioned study, the relationship between endometrium development and progesterone concentration in the mid-luteal phase of women with RM was further analysed (Li *et al.*, 2000) (Table I). Among 33 subjects with delayed endometrial development, 24 had progesterone data on the day of the biopsy, of whom only 33% had suboptimal plasma progesterone (<30 nmol/l), while the remaining 67% of subjects had normal progesterone (⩾30 nmol/l) results. The underlying cause of about two-thirds of subjects with retarded endometrium cannot therefore be explained by suboptimal progesterone stimulation. As the function of the corpus luteum is under the control of the pituitary gland, it is possible that conditions associated with hypothalamo-pituitary dysfunction may also be associated with luteal phase defect, e.g. extremes of weight, extremes of reproductive life, strenuous exercise and stress (Wentz, 1979). However, data in support of these are lacking.

Hyperandrogenaemia

The relationship between hyperandrogenaemia and RM is an interesting one. In a separate study involving serial blood samples obtained on days LH –7, LH –4, LH +0, LH +7 or LH +10, it was found that women with RM ($n = 43$) had higher androgen levels (especially in the follicular phase) than fertile controls ($n = 10$) (Okon *et al.*, 1998). A recent study (Bussen *et al*., 1999) also found that women with RM had higher androstenedione (but not testosterone) levels in the follicular phase than control subjects. In both these studies the presence of hyperandrogenaemia appeared to be independent of the association between polycystic ovarian syndrome (PCOS). Hyperandrogenaemia may be a result of increased androgen production either in the ovary (e.g. polycystic ovary) or the adrenal gland; however, it may also be an indirect consequence of low SHBG, which in turn may be a result of low circulating estrogen levels.

High androgen levels have been negatively correlated with the concentration of PP14, a biochemical marker of endometrial function, in uterine flushings (Okon *et al.*, 1998). Recent in-vitro studies have shown that androgens cause an increase in epidermal growth factor receptor concentration in stromal cells (Watson *et al.*, 1998) and reduce the secretory activities of epithelial cells, resulting in a dose-dependent reduction of PP14 production (Tuckerman *et al.*, 2000) which is consistent with an adverse effect of androgens on endometrial glandular cell function.

Endometrial steroid receptor defect

Initial investigations into levels of estrogen receptors (ER) and PR in the human endometrium utilized radioactive binding assays, and produced conflicting results on the relationship between ER and PR expression and luteal phase defects (Levy *et al.*, 1980; Gravanis *et al.*, 1984; McRae *et al.*, 1984; Hirama and Ochiai, 1995). An immunocytochemical study (Lessey *et al.*, 1996) of 80 women with luteal phase defect (LPD) showed an association between LPD, failure of PR glandular down-regulation and aberrant $\alpha_V\beta_3$ integrin expression, leading the authors to conclude that establishment of normal endometrial receptivity appears to be tightly associated with the down-regulation of epithelial PR. We have been conducting preliminary studies in the use of immunocytochemistry for the identification of a possible endometrial steroid receptor defect as an explanation for the observed abnormality. However, we have found significant variations in staining pattern in different parts of the same biopsy obtained from non-conception cycles (Figure 1e and f) of both normal control subjects and women with RM.

Estrogen receptor

In frozen sections from endometrial biopsies, obtained from non-conception cycles during the luteal phase of the cycle (days 15–28), a steady decrease was observed (Figure 1a–d) in the expression of ER from a mid-cycle peak in both stroma and glandular epithelium. ERα staining was faint at day 23 and virtually absent at day 28; this observation was consistent with previous reports (Lessey *et al.*, 1988; Rogers *et al.*, 1996). In examining ER staining in endometrial biopsies from 29 women with RM at days LH +7 to LH +10 of the cycle, it was found that the majority (26/29) of biopsies appeared normal, whereas three out of 29 biopsies had stronger gland epithelium staining than expected for the date of the biopsy. Two of these biopsies had been classified as in-phase (normal), and one as showing histological features of retarded development by >2 days (LPD). However, it was found that the wide regional variation in the intensity and pattern of staining make identification of abnormal patterns of ERα by immunocytochemistry difficult.

Progesterone receptor

Stromal cell PR immunostaining in endometrial biopsies from luteal phase (days 15–31) of normal, fertile subjects ($n = 11$) were found to show a steady decrease in expression from a mid-cycle maximum. Epithelial cell staining was also maximal at mid-cycle, but showed a more rapid decrease in expression, particularly between days 22 and 24. This menstrual cycle pattern of staining is consistent with previous observations (Press *et al.*, 1988). In contrast to the pattern of positive stroma and negative glandular epithelium seen in the normal control biopsies during the mid to late luteal phase of the cycle, PR-positive gland epithelial cells could be identified in nine out of 25 of the biopsies obtained on days LH +7 to LH +10 from RM patients (Figure 2). Eight of these biopsies were classified as in-phase, and one as out-of-phase (LPD). These data suggest that differences in PR expression may be present in about one-third of women with RM.

The antibody used in the above study located both isoforms of the progesterone receptor, PRA and PRB. PRA is known to repress PRB, ER, glucocorticoid, androgen and mineralocorticoid receptor activity, and the discovery of an inhibitory domain within PRA that is masked by PRB suggests that the two PR isoforms may react with different cellular proteins (Giangrande and McDonnell, 1999). Recent investigations using a dual immunofluorescence technique (Mote *et al.*, 2000) have shown that whilst PRA predominates in secretory stroma, PRB alone may persist in mid-secretory gland epithelium.

Androgen receptor

Immunocytochemical analysis of androgen receptor staining in biopsies from normal fertile women showed its expression mainly in the stroma, which did not change during the cycle. Staining in biopsies from RM patients was largely similar to biopsies from normal fertile women.

In summary, preliminary results using immunohistochemistry suggest that differences in steroid receptor unrelated to LPD may be present in a small subpopulation of women with unexplained RM. However, these studies should be strengthened by further investigations using techniques such as in-situ hybridization, RT–PCR and RNase protection assays which will detect the difference in mRNA expression.

Uterine pathology

Endometrial responsiveness to the circulating steroid hormones may be affected by a number of congenital or acquired uterine pathologies.

Among the various congenital structural uterine anomalies, the septate uterus is the most common. There is little doubt that this is associated with an increased risk of miscarriage due to impairment of implantation (for review, see Homer *et al.*, 2000). It is now recognized that the septum is rather avascular. Scanning electron microscopy was used to compare endometrial biopsy specimens obtained from the septum and the lateral uterine wall in the pre-ovulatory phase (Fedele *et al.*, 1996). These authors found that the septal endometrium showed defective development, indicative of a reduction in the sensitivity to steroid hormones.

Asherman's syndrome is an acquired condition which is due to the presence of post-traumatic intra-uterine adhesions, partly or completely obliterating the uterine cavity. Endometrial responsiveness to steroid hormones is reduced in areas affected by intra-uterine adhesions or fibrosis. A lesser degree of damage to the endometrium may produce patchy fibrosis without a significant amount of intra-uterine adhesion, which is sometimes referred to as partial or incomplete Asherman's syndrome. Successful division of the intra-uterine adhesions in cases without extensive fibrosis may restore the responsiveness of the endometrium and lead to regular menstruation. However, if there are extensive, dense fibrosis the prognosis is poor.

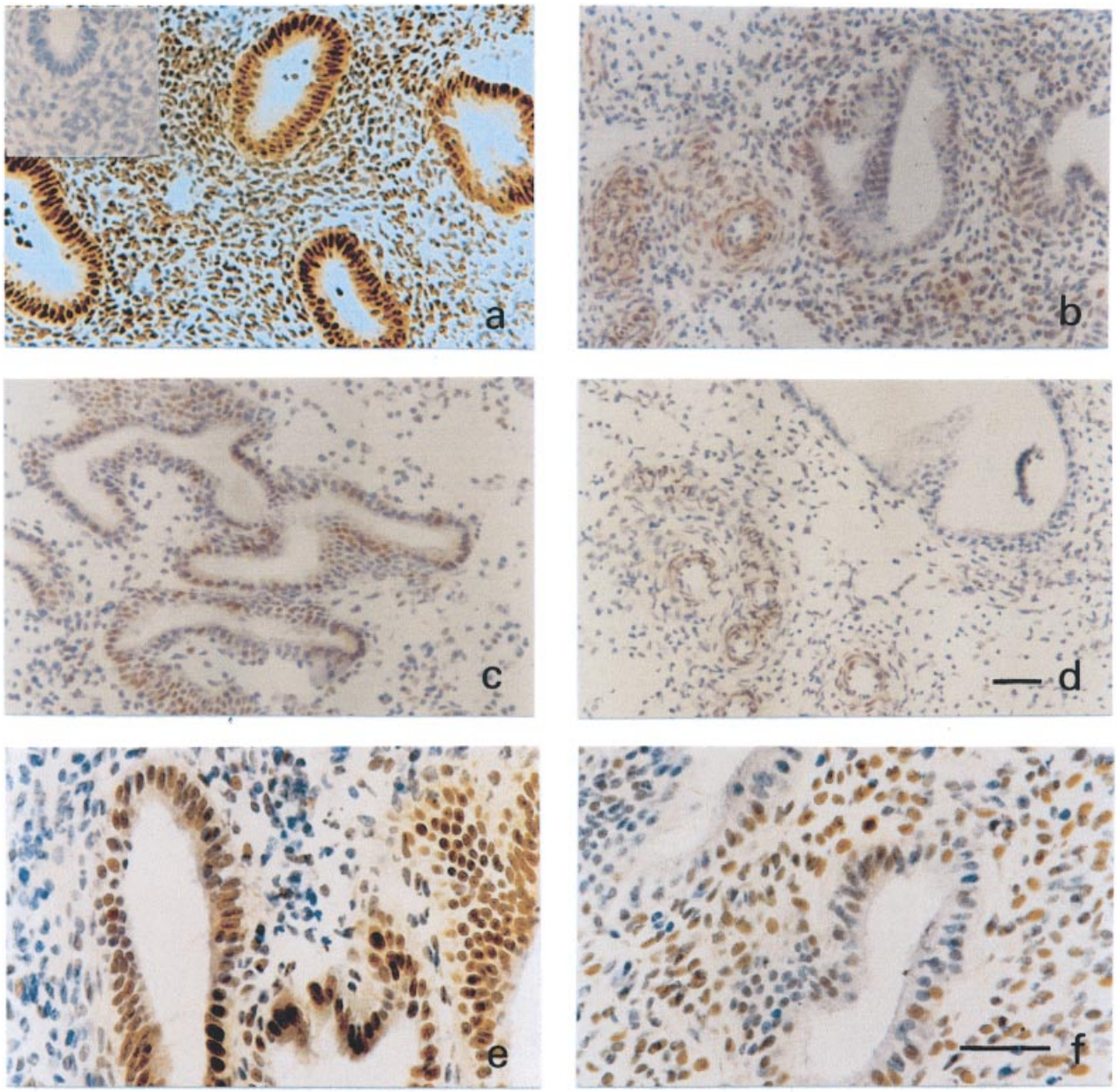

Figure 1. Immunocytochemical staining for estrogen receptor (ER)α in frozen sections of endometrium from normal women and women suffering from recurrent miscarriage (RM). (**a**) Normal mid-proliferative endometrium showing intense brown positive staining in the glandular epithelium and stroma. Insert in upper left-hand corner shows a negative control with the primary antibody omitted. (**b**) Normal mid-secretory endometrium showing pale staining in gland epithelium and stroma, with positive staining around blood vessels. (**c**) Endometrium from RM patient at LH +6 showing increased positive staining in the gland epithelium. (**d**) Endometrium from RM patient at LH +7 showing a normal mid-secretory pattern. Original magnification ×200; scale bar = 50 μm (**e**, **f**) Normal mid-cycle endometrium showing different patterns of ER expression in the same biopsy. Original magnification ×400; scale bar in (f) = 50 μm.

The situation with respect to uterine fibroids—which may also affect implantation and increase the risk of miscarriage—is less clear. There are convincing observational data from five IVF series to suggest that reproductive outcome is significantly compromised with submucous fibroids (i.e. fibroids distorting the cavity), modestly compromised with intramural fibroids, and possibly compromised with subserosal fibroids (Seoud *et al.*, 1992; Farhi *et al.*, 1995; Eldar-Geva *et al.*, 1998; Ramzy *et al.*, 1998; Stovall *et al.*, 1998; Bajekal and Li, 2000). It is not known if the endometrium covering a submucous fibroid or close to an intramural fibroid responds suboptimally to steroid hormones. However, it appears from a number of retrospective and cohort studies that there is good evidence that removal of submucous fibroids reduces miscarriage rate, and some evidence that removal of intramural fibroids also reduces miscarriage rate (Li *et al.*, 1999; Bajekal and Li, 2000). Nevertheless, the improvement in outcome may be produced independent of the endometrial factor.

The prevalence of uterine pathology was examined among 106 women with RM by hysterosalpingogram (HSG) and hysteroscopy (Raziel *et al.*, 1994). Their findings included: HSG, normal

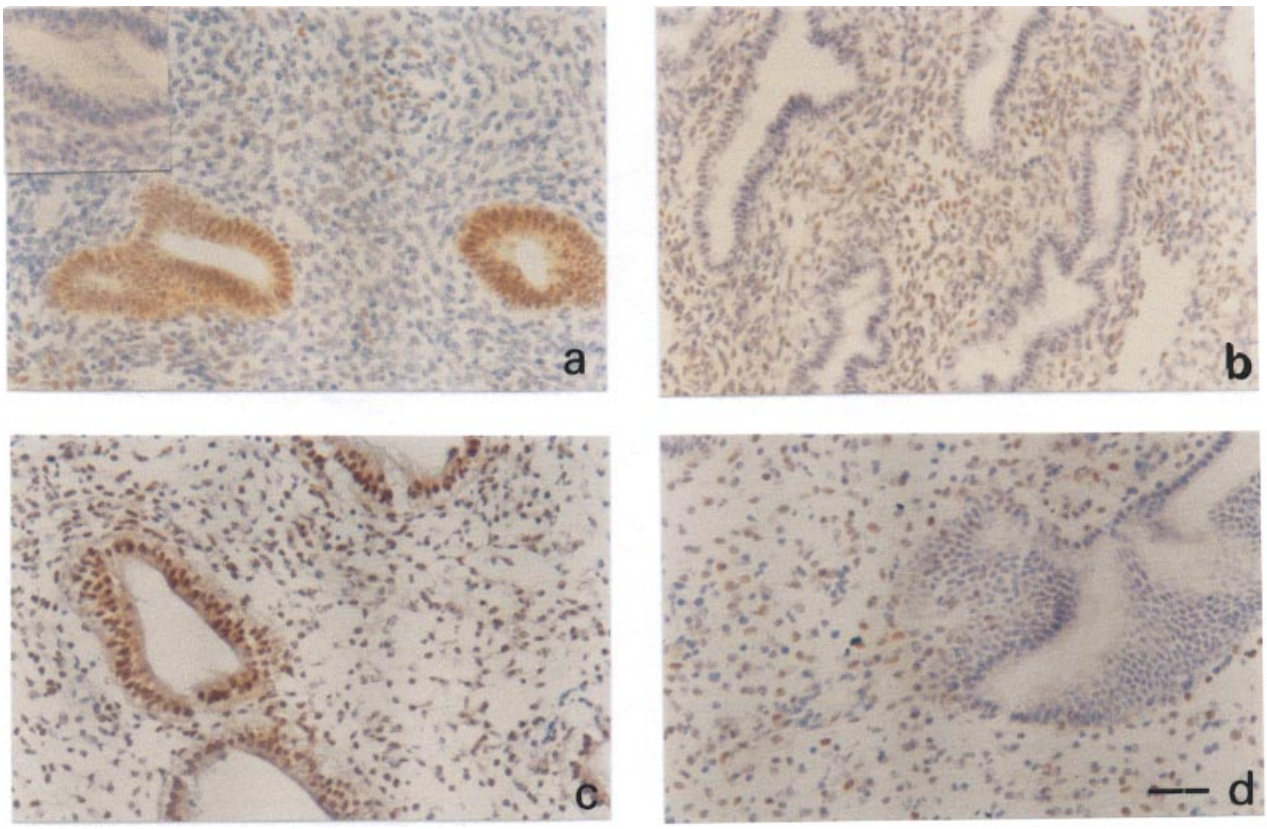

Figure 2. Immunocytochemical staining for progesterone receptor (PR) in frozen sections of endometrium from normal women and women suffering from recurrent miscarriage (RM). (**a**) Normal mid-proliferative endometrium showing brown positive staining in the glandular epithelium and stroma. Insert in upper left-hand corner shows a negative control with the primary antibody omitted. (**b**) Normal mid-secretory endometrium showing the typical pattern of negative gland epithelium and positive stroma. (**c**) Endometrium from RM patient at LH +6 showing positive staining in the gland epithelium. (**d**) Endometrium from RM patient at LH +9 showing a normal mid-secretory pattern. Original magnification ×200; scale bar = 50 µm.

findings 43.6%, uterine septum 17.9% and filling defects and/or uterine wall irregularity 38.7%; hysteroscopy, normal findings 53%, uterine septum 21.7%, intra-uterine adhesions 23.6% and endometrial polyp 1%. HSG is performed routinely in our patients with RM, and a lower prevalence of uterine cavity abnormality (15%) has been found.

Clinical relevance

The clinical value of the observed abnormalities in the endometrium need to be addressed. First, for the observation to provide useful clinical information it should be persistent in subsequent cycles. There has been no formal study to address this question, apart from a small study in five subjects with RM with delayed endometrium (Serle, 1993); in all of these subjects, a repeat endometrial biopsy showed morphological and immunohistochemical abnormalities to be persistent.

Second, for the observed abnormality to be clinically useful, it should be of significant prognostic value. Several studies attempted to examine the prognostic value of the measurement with reference to the success or failure of a subsequent pregnancy. In a preliminary survey (Serle, 1993), it was found that morphological evaluation appeared to be of significant prognostic value in a small group of subjects ($n = 11$). Others (Quenby *et al*., 1999) examined the leukocyte population in endometrial biopsy specimens from women with RM. Those who miscarried again ($n = 10$) following endometrial biopsy had a significantly increased number of at least one of the leukocyte markers CD4+, CD8+, CD14+, CD16+ or CD56+ in their endometrium compared with those who had a subsequent pregnancy resulting in a live birth ($n = 12$). Levels of PP14 in endometrial flushings are also lower in women with RM who subsequently miscarry compared with those who have a live birth (Dalton *et al.*, 1998). However, in both these studies the true prognostic value, i.e. the likelihood of a subsequent pregnancy resulting in live birth among those with normal versus abnormal endometrial leukocytes or PP14 levels, was not formally evaluated. The need to determine the prognostic significance of endometrial cytokine expression was also mentioned by another group (Lim *et al*., 2000), but in this study of relatively small numbers ($n = 25$) there was insufficient power to assess the results.

In summary, it remains uncertain whether any of the methods used to evaluate the endometrium in women with RM have any significant prognostic value, and there is a clear need to address this issue to determine the value of these measurements.

Endometrial versus embryo factors

In a recent study (Li *et al.*, 2000), it was found that the median number of miscarriages in women with RM with delayed endometrium was four, compared with a median of three in women with normal endometrium. Although the difference did

not reach statistical significance, it was of interest to compare these results with those of others (Ogasawara *et al*., 2000), who found that the frequency of abnormal embryonic karyotypes significantly decreased and that of normal karyotypes significantly increased with the number of previous miscarriages. Successful implantation and continuing pregnancy requires dialogue between the embryo and endometrium. Karyotypic analysis detects structural and numerical abnormalities in embryonic chromosomes, and will not identify other genetic defects such as point mutations and homozygosity for deleterious recessive genes. However, the observed increase of normal karyotypes with an increase in the number of miscarriages suggests a correlation between increased miscarriages and the significance of endometrial (maternal) factors.

Treatment of endometrial abnormalities associated with RM

As mentioned earlier, it is uncertain if the association between RM and the abnormality detected by the various measurements of the endometrium is causal or casual. Very few data exist on the value of any treatment on observed endometrial abnormalities. In considering the results of treatment, there are two possible approaches.

First, the impact of treatment on the observed abnormality in the endometrium could be evaluated by repeating the observation during a treatment cycle. A pilot study (Serle, 1993) was carried out to compare the impact of two treatment modalities, progesterone and HMG, among seven subjects on endometrial morphometry and expression of four endometrial proteins (D9B1, 5D4, HMFG1 and BC3), using an immunohistochemistry approach. It appeared from this study that progesterone treatment is of little value, whereas HMG treatment is of possible benefit.

The study was subsequently extended (Li *et al*., 2001) to include 13 subjects who had RM and retarded endometrium. A further endometrial biopsy was obtained in an HMG treatment cycle at 7–10 days after intramuscular administration of an ovulatory dose of HCG (5000 IU). The histological dating of 11 of the 13 (85%) biopsies was within 2 days of chronological dating (i.e. normal), whereas two of the 13 (15%) biopsies were still more than 2 days behind the chronological date (i.e. persistently retarded). The mean (± SD) endometrial dating results (histological dating minus chronological dating) in stimulated cycles was –1.1 ± 1.6 days, compared with –3.5 ± 0.6 days in unstimulated cycles ($P < 0.05$).

Second, the benefit of any proposed treatment, on clinical outcome, that is improving the chance of a subsequent pregnancy resulting in a live birth, needs to be examined. A study was conducted to examine the impact of HMG treatment on the outcome of a subsequent pregnancy (Li *et al.*, 2001). Women with unexplained RM associated with retarded endometrial development in the peri-implantation period were allocated to two groups: (i) treatment with HMG; and (ii) a non-treatment group. Thirteen pregnancies arose from group (i) and 12 pregnancies occurred in group (ii). The live birth rate in the treatment group (11/13 = 85%) was higher than that of the non-treatment group (5/12 = 42%).

The results presented above are clearly preliminary. However, the encouraging data would appear to support the case for a larger-scale clinical trial to test the hypothesis that controlled ovarian stimulation is an effective treatment of endometrial abnormality observed in women with unexplained RM. Nevertheless, based on the current data, power calculation indicates that the number of patients required in a prospective randomized control trial is 250 subjects, assuming that the live birth rate in the treatment group is 80% and in the control group is 40%, and assuming that the prevalence of endometrial abnormality is 20% among women with recurrent miscarriage, and accepting errors of $\alpha = 0.05$ and $\beta = 0.8$. Further, if the actual live birth rate in the control group is slightly higher than expected (e.g. ~50%), the number of subjects required would need to be increased to 400 (Li, 1998). Almost certainly, a multicentre study will be required to address this question formally.

Summary

An endometrial factor does appear to exist in RM. Some cases might be due to endocrine abnormality or intra-uterine pathology, but in at least 50% of cases the underlying cause is not known. The clinical implications of the observations including prognosis and treatment have yet to be established.

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