Phenotypic and functional studies of leukocytes in human endometrium and endometriosis

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The aetiology of endometriosis, a common and disabling disorder, is presently unknown, although immune dysfunction could allow ectopic endometrial fragments to survive outside the uterine cavity. These studies investigate the relationship between leukocyte populations, steroid hormone receptor expression, proliferative activity, bcl-2 expression and apoptosis in eutopic and ectopic endometrium from women with endometriosis or adenomyosis at different phases of the menstrual cycle. Significantly increased oestrogen receptor expression, bcl-2 expression and numbers of CD8+ leukocytes were found in ectopic compared with eutopic endometrium in endometriosis, and CD56+ endometrial granulated lymphocytes (eGLs) were significantly reduced in ectopic endometrium. Apoptotic cells were rarely found in control and subject endometria. In contrast with endometriosis, adenomyotic lesions showed identical steroid hormone receptor expression, proliferative activity, bcl-2 expression and leukocyte subpopulations to eutopic endometrium, indicating different aetiologies for these disorders. The unusual CD56+ CD16– eGLs present in large numbers in late secretory phase eutopic endometrium were highly purified (>98%) by immunomagnetic separation. Except for a negligible cytotoxic activity of eGLs from early proliferative samples, cytotoxic activity of eGLs from non-pregnant endometrium during the menstrual cycle was comparable with those in peripheral blood, predominantly CD56+ CD16+ natural killer cells. eGLs from non-pregnant endometrium and early pregnancy showed a variable proliferative response to 5 and 100 U/ml interleukin-2 over 48-h and 120-h time courses. eGLs are evidently functionally important in the eutopic endometrium. Their absence in endometriotic lesions together with increased CD+8 T-cell numbers and increased oestrogen receptor and bcl-2 expression may have significant effects on the development and progression of endometriosis.

Key words: cytotoxicity/endometriosis/endometrium/leukocytes/proliferation

Introduction

Endometriosis is a common and disabling gynaecological disorder often associated with infertility. Its aetiology is currently unknown, although it has been proposed that immune dysfunction may be involved. Numerous studies have highlighted that several components of the immune system are altered in women with endometriosis. Many studies have focused on the role of natural killer (NK) cells, with the majority showing some degree of NK-cell dysfunction (Oosterlynck et al., 1991, 1992; Vigano et al., 1991; Tanaka et al., 1991; Tanaka et al., 1992; Gurzetti et al., 1993, 1995; Iwasaki et al., 1993; Wilson et al., 1994; Ho et al., 1995). Others have demonstrated increased numbers of activated macrophages in women with endometriosis (Oosterlynck et al., 1994; Becker et al., 1995). These studies suggest a role for altered cell-mediated immunity in the development of endometriosis, but the exact mechanism by which this is mediated remains unclear.

Normal eutopic endometrium contains numerous leukocytes in both stromal and intraepithelial locations.
(King et al., 1989; Bulmer et al., 1991; Pace et al., 1991). In endometrial stroma, the number of leukocytes increases from the proliferative phase, where they account for ∼ 5–10% of the total stromal cell population, to the late secretory phase where ∼ 20–25% of stromal cells are leukocytes (Bulmer et al., 1991; Jones et al., 1996). This large increase in the endometrial leukocyte population is due to an increase in the phenotypically unusual population of CD56+ CD16- endometrial granulated lymphocytes (eGLs) (Bulmer et al., 1991). Human endometrium clearly contains numerous leukocytes which are changing throughout the menstrual cycle. Since endometriosis may occur as the result of immune dysfunction, it is surprising that few studies have addressed the possibility that altered leukocyte populations in either eutopic or ectopic endometrium are involved in disease aetiology and pathogenesis.

Adenomyosis, characterized by the presence of endometrial glands and stroma within the uterine myometrium, is generally thought to occur as a result of migration of endometrium from the stratum basalis, but its aetiology remains unclear (Garcia et al., 1987; Ryan et al., 1990; Fox and Wells, 1995). Our investigations have focused on the leukocyte subpopulations in eutopic and ectopic endometrium from women with either ovarian endometriosis or adenomyosis throughout the menstrual cycle. These studies have addressed the factors which may influence or regulate the leukocyte populations within the endometriotic lesions, such as steroid hormone receptor expression, proliferative activity, bcl-2 expression and apoptosis.

Although it has been suggested that NK cells play an important role in the aetiology of endometriosis, several studies have shown that CD56+ CD16+ ‘classical’ NK cells are only present in negligible numbers in eutopic and ectopic endometrium (Bulmer et al., 1991; Klentzeris et al., 1992; Oosterlynck et al., 1993; Fernández-Shaw et al., 1995; Jones et al., 1996; Lachapelle et al., 1996). In contrast, the unusual CD56+ CD16- eGLs are present in large numbers in eutopic endometrium, but their presence in ectopic endometrium has received scant attention. Although there have been several studies of the function of eGLs in early pregnancy, the role and function of eGLs in the non-pregnant endometrium have not been addressed. These cells may prove to have a crucial role in normal endometrium and in the pathogenesis of endometriosis.

### Stromal leukocyte subpopulations

Several workers have reported that ectopic endometrium from primarily ovarian endometriotic lesions contained elevated numbers of leukocytes, predominantly CD3+ T-cells, and reduced numbers of macrophages (Klein et al., 1992, 1994; Witz et al., 1994; Jones et al., 1996). However, others have failed to detect any differences in the leukocyte populations between the eutopic and ectopic endometrium (Fernández-Shaw et al., 1995).

In order to further assess changes in endometrial leukocyte populations with menstrual cycle phase in endometriosis, the leukocyte populations in formalin-fixed paraffin-embedded sections of eutopic and ectopic endometrium from women with either primarily ovarian endometriosis (n = 30: proliferative n = 10, early secretory n = 10, late secretory n = 10) or adenomyosis (n = 15: proliferative n = 5, early secretory n = 5, late secretory n = 5) were examined using a streptavidin–biotin immunohistochemical technique as described in Jones et al. (1996). Leukocyte populations in endometriosis and adenomyosis were also compared with those in control endometrium (n = 30: proliferative n = 10, early secretory n = 10, late secretory n = 10) at different phases of the menstrual cycle. The findings are presented in Figure 1. There were no significant differences in leukocyte populations when control and eutopic endometrium from women with endometriosis were compared. Comparison of leukocyte populations in eutopic and ectopic endometrium from women with endometriosis, however, showed a significantly lower percentage of CD56+ cells as a proportion of the LCA+ cells in ectopic endometrium at all phases of the menstrual cycle. In contrast, the percentage of CD3+ cells was elevated in ectopic endometrium, although this was not statistically significant. Similarly, the percentage of CD8+ cells was higher in ectopic than in eutopic endometrium at all phases of the menstrual cycle, although this was statistically significant only in the late secretory phase. The percentage of CD4+ cells and macrophages were generally similar in eutopic and ectopic endometrium. Additionally, the absolute number of CD56+ cells was reduced and the absolute numbers of CD3+ and CD8+ cells were significantly higher in ectopic endometrium than in eutopic endometrium.

Leukocyte populations were similar in eutopic and ectopic endometrium from women with adenomyosis at all phases of the menstrual cycle. Furthermore, the leukocytes in the eutopic endometrium did not differ significantly from those in either control endometrium or ectopic endometrium from women with endometriosis. In adenomyotic lesions, however, there were fewer LCA, CD3, CD8 and CD68+ cells and more CD56+ cells than in endometriotic lesions at all phases of the menstrual cycle (Figure 2).
These findings extend previous studies of endometrial leukocyte subpopulations in normal and pathological endometrium by taking into account the effect of menstrual cycle phase on leukocyte populations. Interestingly, ectopic endometrium in endometriotic lesions contains altered leukocyte subpopulations compared with the paired eutopic endometrium from the same patient and with ectopic endometrium from adenomyotic lesions. Our findings suggest that ectopic endometrium in endometriotic lesions contains a similar leukocyte component to proliferative-phase eutopic endometrium. While the reasons for this remain unclear, these changes in leukocyte populations support the proposal that alterations in the cell-mediated immune system may have a role in disease aetiology and pathogenesis. As these differences in leukocyte populations were not noted in eutopic endometrium, it seems unlikely that endometriosis results from alterations in the eutopic endometrium which make the endometrial fragments more likely to develop into endometriotic lesions. There is the proviso that these changes in leukocyte populations may be an epiphenomenon occurring as a result of the endometrial tissue being present in an ectopic location. Altered leukocyte populations within the ectopic endometrium may secrete abnormal levels of cytokines and growth factors with growth-promoting and angiogenic properties, thereby affecting the growth and development of the endometriotic lesions.

The significant reduction of eGLs in ectopic endometrium in endometriosis is a striking finding in view of the proposed role of ‘classical’ NK cells in the pathogenesis of endometriosis. It is clearly important to clarify the role of eGLs in eutopic endometrium and to assess the possible effects of eGL absence on the development of endometriotic lesions. The marked differences in leukocyte populations between adenomyotic and endometriotic lesions highlight that these disorders, which are characterized by the presence of ectopic endometrial tissue, are likely to have different aetiologies.

Intraepithelial leukocyte subpopulations
Leukocytes are also present in intraepithelial locations in eutopic and ectopic endometrium. Pace et al. (1991)
reported that intraepithelial leukocytes (IELs) were present in similar proportions to those in endometrial stroma. We have examined IELs in eutopic and ectopic endometrium in endometriosis and adenomyosis. The changes in IEL populations were similar to those for stromal leukocytes, namely increased CD8+ intraepithelial T-cells and reduced numbers of CD56+ IELs in endometriotic lesions compared with IELs in eutopic endometrium. Our study highlights the close association between stromal leukocytes and IELs and raises the possibility that alterations in IEL populations in endometriotic lesions may play a role in the aetiology of endometriosis. There were no significant differences between eutopic endometrium from normal women and women with endometriosis; these findings, therefore, do not indicate a role for IELs in the infertility associated with endometriosis.

**Steroid hormone receptor expression**

It is important to define the factors which may account for the altered leukocyte subpopulations within endometriotic lesions. As oestrogen and progesterone are the hormones most likely to regulate changes in leukocyte populations during the menstrual cycle and endometriosis is an oestrogen-dependent disease (Schenken, 1989), oestrogen and progesterone receptor expression in endometriotic lesions was studied (Jones et al., 1995). Epithelial and stromal oestrogen receptor expression was significantly increased in endometriotic lesions throughout the menstrual cycle. Progesterone receptor expression, however, was similar in eutopic and ectopic endometrium. Double immunohistochemical labelling studies revealed that leukocytes do not express either oestrogen or progesterone receptors in eutopic and ectopic endometrium and were not responsible for the increased oestrogen receptor expression.

Since ectopic endometrium in endometriosis contains a leukocyte profile similar to that of proliferative phase endometrium and oestrogen is the predominant steroid hormone present during the proliferative phase, it is reasonable to speculate that the altered leukocyte populations in endometriotic lesions may result from enhanced effects due to their high levels of oestrogen receptors. In support of this notion we have recently demonstrated that eutopic and ectopic endometrium from women with adenomyosis contained comparable levels of oestrogen and progesterone receptors. Moreover, when oestrogen and progesterone receptor expression was compared between ectopic endometrium from endometriosis or adenomyosis, epithelial progesterone receptor expression was identical in both tissues, although stromal progesterone receptor expression tended to be higher in ectopic endometrium in adenomyosis. Stromal and epithelial oestrogen receptor expression, however, was lower in ectopic endometrium in adenomyosis at all phases of the cycle except in late secretory phase stroma (Figure 3). These findings suggest that the altered leukocyte subpopulations in endometriotic lesions may result from up-regulated oestrogen receptor expression, thereby supporting a role for altered oestrogen receptor expression in the aetiology and pathogenesis of endometriosis.

**Proliferative activity**

Endometriosis and adenomyosis are characterized by abnormal growth of endometrial tissue; it is therefore important to study the proliferative activity of epithelial and stromal cells within these tissues. We reported that ectopic endometrium in endometriosis had lower proliferative activity, although there were no differences in the proliferative activity between eutopic and control endometrium (Jones et al., 1995). These findings are in agreement with those of other workers (Klein et al., 1992, 1994; Wingfield et al., 1995; Jürgensen et al., 1996) but conflict with the study of Li et al. (1993) who reported increased proliferative activity in ectopic endometrium in endometriosis.

These studies have been extended to compare the proliferative activity of eutopic and ectopic endometrium from women with adenomyosis with that of control...
endometrium and eutopic and ectopic endometrium in endometriosis. Eutopic endometrium in adenomyosis displayed similar proliferative activity to that of control endometrium except in proliferative and early secretory phase stroma. Moreover, there were no significant differences in epithelial and stromal proliferative activity between eutopic and ectopic endometrium in adenomyosis nor between ectopic endometrium from women with endometriosis or adenomyosis. These findings suggest that altered proliferative activity is unlikely to play a major role in the aetiology of either endometriosis or adenomyosis.

Bcl-2 expression and apoptosis

Studies of endometrium in non-human mammals have indicated that apoptosis is important in the regulation of normal endometrial function (Nawaz et al., 1987; Pollard et al., 1987; Moulton, 1994). Few studies, however, have examined apoptosis in human endometrium. If apoptosis is important in the control of normal endometrium then any dysfunction may have a role in the aetiology of endometriosis. Recent research has identified that several factors, including bcl-2 (a proto-oncogene product), regulate apoptosis (Hockenbery et al., 1990). Moreover, a close correlation exists between bcl-2 expression and oestrogen and progesterone receptor expression in human endometrial epithelium (Gompel et al., 1994; Otsuki et al., 1994; Koh et al., 1995; Tabibzadeh et al., 1995; Harada et al., 1996; McLaren et al., 1997). In contrast, bcl-2 expression in endometrial stroma has received scant attention (Gompel et al., 1994; Koh et al., 1995; McLaren et al., 1997).

We investigated whether altered apoptosis may be involved in the pathogenesis of endometriosis. We speculated that up-regulated oestrogen receptor expression may increase bcl-2 expression, down-regulate apoptosis and prolong the life of cells within the endometriotic lesions, thereby accounting for the development of the disease.

Apoptosis was examined in formalin-fixed paraffin-embedded sections of control, eutopic and ectopic endometria from women with either endometriosis or adenomyosis at different phases of the menstrual cycle using the TUNEL Apoptosis Detection System (Promega, Southampton, UK). Negligible numbers of apoptotic epithelial and stromal cells were detected in control and subject endometrial samples. This finding is in agreement with other studies that have indicated that apoptosis does not have a significant role in the control of proliferation in

![Figure 4. Comparison of the percentage of bcl-2+ stromal cells in eutopic and ectopic endometria in endometriosis or adenomyosis at different phases of the menstrual cycle. *P < 0.05 > 0.01, **P < 0.01 > 0.001.](image)

the human endometrium (Tabibzadeh et al., 1994; Yasuda et al., 1995; Harada et al., 1996).

Unlike bcl-2 expression in control endometrial epithelium which declined after the proliferative phase, bcl-2 expression in stroma was highest in the late secretory phase. There were no differences in bcl-2 expression between eutopic endometrium from controls and patients with endometriosis or adenomyosis and in epithelial bcl-2 expression between eutopic and ectopic endometrium in endometriosis or adenomyosis; in endometriosis there were significantly more bcl-2+ cells in ectopic than in eutopic endometrial stroma in the proliferative and early secretory phases (Figure 4). In contrast, bcl-2 expression was identical in eutopic and ectopic endometrium in adenomyosis (Figure 4).

Since there are leukocytes present in endometriotic lesions, the identity of the bcl-2+ cells in eutopic and ectopic endometrium was investigated by double immunohistochemical labelling. In eutopic endometrium, the majority of bcl-2+ cells were CD45+ leukocytes; the bcl-2+ cells were CD68 negative, that is, not macrophages. Ectopic endometrium contained numerous bcl-2+ leukocytes as well as a considerable population of bcl-2+ stromal cells which was not detected in eutopic endometrium. This novel finding suggests a possible role for endometrial bcl-2+ stromal cells in the development of endometriosis. The prolonged survival of bcl-2+ stromal cells in endometriotic lesions may account for the growth of the lesions as well as the secretion of cytokines and growth factors which regulate the function of the leukocytes within the developing endometriotic lesion.
Endometrial granulated lymphocyte function

eGLs are an important and dynamic population in eutopic endometrium but their function in non-pregnant endometrium has received scant attention. As eGLs are present in endometriotic lesions in greatly reduced amounts, it is first necessary to investigate their function in normal endometrium in order to address whether low levels of these unusual cells may promote the growth and development of endometriotic lesions.

Cytotoxic activity

eGLs have been proposed to be of NK-cell lineage because of their expression of CD56; at present, however, the exact relationship between peripheral blood, predominantly CD56+ CD16+ NK cells and endometrial CD56+ CD16– eGLs is unclear (King and Loke, 1991; King et al., 1991). Studies of eGLs purified from early pregnancy decidua have reported cytotoxic activity lower than or comparable with that of peripheral blood NK cells (King et al., 1989; Manaseki and Searle, 1989; Ritson and Bulmer, 1989; Ferry et al., 1990; King and Loke, 1990; Saito et al., 1993b; Loke and King, 1995). We therefore compared the cytotoxic activity of eGLs highly purified (>98% CD56+) from endometrium at different phases of the menstrual cycle (n = 33) with CD56+ peripheral blood NK cells (n = 8).

There were no significant differences between the cytotoxic activity of eGLs from late proliferative, early secretory, late secretory and menstrual phase non-pregnant endometrium and CD56+ cells from peripheral blood (Figure 5). In contrast, eGLs from the early proliferative phase had negligible cytotoxic activity which was significantly lower than that of eGLs from other phases of the menstrual cycle and CD56+ cells from peripheral blood.

The high level of eGL cytotoxic activity after the early proliferative phase of the menstrual cycle suggests these cells have an important role in non-pregnant endometrium and may be involved in the protection of the endometrium against infection.

Proliferation to interleukin-2

Although decidual eGLs have been shown to be activated by interleukin-2 (IL-2) (Ritson and Bulmer, 1989; Ferry et al., 1990; King and Loke, 1990; Starkey, 1991; King et al., 1992; Saito et al., 1993b), the capacity of eGLs from the non-pregnant endometrium to respond to IL-2 is unknown. Our study assessed the effect of low (5 U/ml) and high (100 U/ml) levels of IL-2 on the proliferation of eGLs from different phases of the menstrual cycle (n = 27) over long (120 h) and short (48 h) time courses. The results of proliferation in response to 100 U/ml IL-2 are presented in Figure 6; the results of eGL proliferation with 5 U/ml IL-2 were similar to those for 100 U/ml.

eGLs proliferated in response to IL-2, although the response between different samples was variable. There were no significant differences in the proliferative responses of endometrial CD56+ cells in the presence of either 5 or 100 U/ml IL-2 after 48 h or 120 h. The ability of eGLs to respond to IL-2 was not affected by menstrual cycle phase. Interestingly, eGL proliferation occurred over a short time period and with low levels of IL-2. The significance of these findings in vivo is not clear since there is no evidence for the presence of IL-2 within normal endometrium (Saito et al., 1993a; Jokhi et al., 1994; King et al., 1995). The present study indicates that eGLs display...
lymphoproliferative and cytotoxic activity within the normal endometrium during the menstrual cycle and therefore their absence in endometriotic lesions may have important consequences for endometrial dysfunction.

Summary

Our studies have investigated the relationship between leukocyte populations, oestrogen and progesterone receptor expression, proliferative activity, apoptosis and bcl-2 expression in endometriotic and adenomyotic lesions. Increased oestrogen receptor expression and bcl-2 expression in endometriotic lesions may account for the differences in leukocyte subpopulations in ectopic endometrial tissue in endometriosis. The findings suggest that endometriosis and adenomyosis have different aetiologies. Our functional studies have shown that endometrial eGLs are capable of lymphoproliferative and cytotoxic activity and are likely to be concerned with endometrial defence. Their absence from endometriotic lesions may have significance for the development and the progression of the disorder.

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