The distribution of endometrial leukocytes and their proliferation markers in trimegestone-treated postmenopausal women compared to the endometrium of the natural cycle: a dose-ranging study

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The effect of trimegestone-based sequential hormone replacement therapy (HRT) on the distribution of endometrial leukocytes and Ki-67 expression was investigated and the findings compared with the endometrium of the natural cycle. Endometrial cells positive for CD45+, CD56+, CD3+, Ki-67+ and CD45+/Ki-67+ antigens were immunohistochemically evaluated in samples from postmenopausal women who completed a randomized, double-blind, dose-ranging study of oral trimegestone (0.05, 0.1, 0.25 and 0.5 mg per day) from days 15 to 28 with continuous micronized oestradiol 2 mg daily for six treatment cycles. The control samples were luteinizing hormone (LH)-dated endometrial biopsies. Cell counts were interpreted using linear discriminant analysis and unpaired t-test. The dose of trimegestone did not significantly affect the mean count of CD45+, CD56+, CD3+, Ki-67+ and CD45+/Ki-67+ cells in the endometrial biopsies obtained from treated women. Endometrial sections from women who bled on the day of the biopsy contained higher numbers of CD45+ and CD56 cells. In the trimegestone-treated endometrium, CD45+, CD56+ and CD3+ cell expression was similar to the proliferative and early secretory phases of the natural cycle. However, the expression Ki-67 and CD45+/Ki-67+ cells was similar to the menstrual phase of the natural cycle endometrium. Women treated with four doses of trimegestone exhibited four different bleeding patterns. Therefore the endometrial infiltration with these cells did not explain the pattern of bleeding in women on this HRT regimen.

Key words: endometrial leukocyte/hormone replacement therapy/postmenopause/trimegestone

Introduction

The long-term use of hormone replacement therapy (HRT) improves quality of life, prevents postmenopausal bone loss, reduces cardiovascular deaths, and more recently has been reported to reduce the incidence of Alzheimer’s disease (Ross et al., 1981; Daly et al., 1996; Paganini and Henderson, 1996). Unscheduled and heavy uterine bleeding ranks among the most common causes for discontinuation of HRT in postmenopausal women (Nachtigall, 1990). We have demonstrated that an increase in the dose of trimegestone in a sequential HRT regimen can dramatically influence the pattern of bleeding; however, 96% of the endometrial samples obtained at the end of 6 months of treatment with four doses of this progestin were in the secretory phase (Al-Azzawi et al., 1999), and therefore the mere diagnosis of secretory endometrium is not adequate to predict or explain the different patterns of bleeding experienced by patients on this sequential HRT regimen. Similar conclusions were reported (Habiba et al., 1998) when the pattern of bleeding in women receiving sequential norethisterone-based HRT could not be explained by the difference in histological parameters detected in the HRT-treated endometrium, compared to that of the natural cycle.

Endometrial leukocytes account for 10–15% of the stromal cells (Kamat and Isaacson, 1987). The major cell population of leukocytes (as defined by leukocyte common antigen, LCA positive) in the secretory endometrium includes T lymphocytes, endometrial granular lymphocytes (eGL), and macrophages (Kamat and Isaacson, 1987; Song et al., 1996). Progesterone modulates the leukocytic infiltration into the endometrium of the natural cycle (Hameed et al., 1995; Inuoe et al., 1996), where there is an increase in the number of the stromal leukocytes from the early secretory phase onwards. Endometrial leukocytes may be involved in regulating implantation. When this does not occur, these leukocytes may become activated to initiate endometrial disintegration and shedding (Kamat and Isaacson, 1987), due to the release of cytotoxic mediators such as H2 O2 and tumour necrosis factor (TNF)α by the macrophages (Clark and Daya, 1990), for example, while CD56+ cells are reported to produce the cytolytic substance, perforin (Hameed et al., 1995).

The origin of these leukocytes has been disputed: they may be migrating from the circulation under the effect of chemotactic factors produced by stromal cells, or they may be proliferating locally at the endometrium in the beginning of the secretory phase. Ki-67 is a non-histone proliferation-associated antigen which is expressed in all active parts of the cell cycle, but is absent in the resting cell, G0 (Gredes et al., 1984). The detection of Ki-67 protein is used to assess proliferation as it is not degraded during apoptosis. Moreover, endometrial expression of Ki-67 antigen appears to be modulated by sex steroids (Shiozawa et al., 1996).

We examined the leukocytic infiltration and the proportion of proliferating leukocytes in the endometrium of postmenopausal women treated with one of four doses of trimegestone-based HRT, in an attempt to find a dose-related effect of this new nor-pregnane progestogen, and compared the findings to those of the endometrium of the natural cycle.
Figure 1. Immunohistochemical staining of endometrial sections, showing (a) the distribution of CD45<sup>+</sup> cells in the stroma with an aggregation around the gland (arrowhead); (b) CD56<sup>+</sup> cells in the stroma, around the gland (large arrowhead) and intra-epithelial leukocytes (IEL) (small arrowhead); (c) CD3<sup>+</sup> cells in the stroma; (d) Ki-67<sup>+</sup> cells in the stroma and glands; (e) double labelling, CD45<sup>+</sup> cells (large arrowhead), Ki-67<sup>+</sup> cells (small arrowhead), and CD45<sup>+</sup>/Ki-67<sup>+</sup> cells (arrow) in the stroma; and (f) CD45<sup>+</sup>/Ki-67<sup>+</sup> cells with an aggregation around the gland. G = gland. Scale bars in panels a,c,d,e,f, = 40 µm, and in panel b = 70 µm.

Materials and methods
In a randomized, double-blind, dose-ranging study, 176 postmenopausal women were randomized to one of the four doses of oral trimegestone (0.05, 0.1, 0.25 and 0.5 mg per day) from days 15 to 28 with continuous micronized oestradiol 2 mg daily for six treatment cycles. The protocol was approved by the local ethics committee and all patients signed informed consent.

Women included in the study were healthy, aged 45–65 years, with intact uterus who were at least 6 months postmenopausal, had had HRT for 2 years, or had been on HRT for at least 1 year, with pre-treatment follicle stimulating hormone (FSH) and oestradiol concentrations in the postmenopausal range. None had received any form of sex steroid treatment for 6 weeks before the commencement of the study. Those who had ever used oestradiol implants were excluded. Tests for liver and renal function were performed and those women with abnormalities were excluded. All women over the age of 50 years had had a normal mammogram within 3 years, and normal cervical smears within the previous 6 months. General, breast, and pelvic examinations were conducted to confirm normality.

Endometrial biopsies were obtained on day 24 of the last treatment...
Endometrial leukocytes in trimegestone-treated postmenopausal women

The distribution of stromal endometrial leukocytes in the natural cycle.

The distribution of Ki-67$^+$ cells in the endometrial glands and stroma in the natural cycle.

The endometrial samples were fixed immediately in 10% formal–saline, embedded in paraffin wax; and 4 µm sections were stained with haematoxylin and eosin for histological assessment. Control samples were deep endometrial biopsies obtained either from healthy women undergoing laparoscopic sterilization using sharp curette, or from hysterectomy specimens none of which was indicated for menstrual disorder; none of these women received any hormonal treatments for 2 months prior to the procurement of the specimens ($n = 28$) All women were given urinary LH surge detection kits (First Response, Carter Wallace Limited, Folkstone, UK). The biopsies were proliferative ($n = 6$), early secretory ($n = 7$), late secretory ($n = 7$), and menstrual ($n = 8$). These biopsies were dated both by LH surge and the date of the last menstrual period, and were examined by two independent pathologists (B.Hamid, Trafford General Hospital, Manchester, UK and S.Dean, West Suffolk Hospital, Bury St Edmunds, UK), who were blinded to the LH surge and menstrual dates.

**Antibodies**

Mouse monoclonal anti-human antibodies against: (i) Ki-67 (MiB-1 antibody, 1:150, Pharmingen, San Diego, California, USA), (ii) leukocyte common antigen (LCA, CD45 antigen, 1:150, Dako, Glostrup, Denmark), (iii) CD56 antigen (1:25, Novocastra, Newcastle upon Tyne, UK), and (iv) rabbit polyclonal anti-human CD3 antibody (1:150, Dako).

**Immunohistochemistry**

Endometrial sections, 4 µm thick, were incubated in a microwave oven (750 W) in citrate buffer at pH 6.0, for 30 min, for antigen retrieval of Ki-67 and CD56. Pepsin digestion (0.4%) was used for antigen retrieval of CD45 (20 min) and CD3 (40 min). Sections were then incubated with normal rabbit serum (NRS; Dako) or normal swine serum (NSS, Dako for CD3) to minimize non-specific reactivity. Specimens were washed with phosphate-buffered saline (PBS) for 20 min between the steps. The sections were then incubated with the relevant primary antibody, overnight at room temperature for Ki-67 and CD56 or at 4°C for CD45 and CD3. Endometrial sections were incubated with species specific biotin-linked secondary antibody (Dako), vectastain ABC peroxidase, and DAB substrate (diaminobenzidine; Vector Laboratory, Peterborough, UK) according to the manufacturers’ recommendations.

Double labelling of the endometrial sections with anti-CD45 and Ki-67 was performed to demonstrate the proportion of the proliferating leukocyte (CD45$^+$/Ki-67$^+$). Steps were followed for CD45 antigen detection using secondary antibody linked to alkaline phosphatase substrate (Fast red TR/naphthol AS/MX, Sigma, Dorset, UK), followed by Ki-67 staining for which the secondary antibody linked to blue/grey peroxidase substrate was used (SG, Vector Laboratory, Peterborough, UK). Negative controls were prepared by replacing the primary antibody with NRS (NSS for CD3 antigen).

**Assessment of the staining**

The pattern of the distribution of the positively stained cells for all the antigens was assessed in the endometrial slides, and then 10 randomly selected fields (Hamilton, 1995) per slide (magnification ×200) were captured to evaluate the endometrial cells positive for CD45, CD56, CD3 and Ki-67 antigens. In the doubly labelled sections and the LH-dated control endometrial specimens, 15 randomly selected fields per slide were captured. Images were captured using an Axioplan microscope (Carl Zeiss, Herts, UK), and a colour video camera (Sony CCD/RGB); each image (magnification ×200) covers an area of 0.121 mm$^2$ using an eyepiece graticule. The images were displayed on an RGB (red, green, blue) monitor and the positive cells
Figure 5. Linear discriminant analysis showing the distribution of positively stained cells in the trimegestone-treated endometrium and their relationship to the phases of the natural cycle for antigens: (A) CD45\(^+\) cells, (B) CD56\(^+\) cells, (C) CD3\(^+\) cells (D) Ki-67\(^+\) cells and (E) CD45\(^+\)/Ki-67\(^+\) cells. Light crosses represent the mean cell count in the treated individual women and the heavy crosses represent the group means. Open circles show the mean cell count in the endometrium of the natural cycle. Solid circles represent the group means: P = proliferative; ES = early secretory; LS = late secretory; M = menstrual.

were counted using the KS300 image analysis program (Kontron Imaging Systems, Thame, UK). Cell counts were restricted to the functionalis layer.

Statistics
Cell counts in all randomized fields, for each marker used, were analysed using a linear discriminant analysis model based on eight groups, viz. the four doses of the trimegestone-treated specimens, and the four phases of the endometrium of the natural cycle (proliferative, early secretory, late secretory and menstrual) (GENSTAT 5.3 package; GENSTAT 5 committee 1993; Mardia et al., 1979). Unpaired t-test was used to compare the cell count of specimens obtained from women who bled on the day of the biopsy with those who had not. Based on assumed SD of 40 for the CD45 cell count, and 20 for the CD3 and CD56 cell counts, these tests had 80% power to detect a mean difference (at \(\alpha = 0.05\)) of 26 and 13 respectively.

Results
Of 176 women included in the initial randomization, 131 completed the study. Seven women did not start treatment after randomization and were withdrawn from the study because they were lost to follow-up (\(n = 3\)), made a protocol violation (\(n = 3\)), or adverse events occurred (\(n = 1\)). A further 38 women did not complete the study, of whom only nine withdrew due to irregular bleeding. There was no statistical difference between the number of patients who withdrew from each trimegestone dose group. Age, duration of the menopause, previous use of HRT, height, weight, or body mass index did not influence the bleeding pattern among the four trimegestone dose groups.

Endometrium of the natural cycle
CD45\(^+\), CD56\(^+\) and CD3\(^+\) cells in the endometrium of the natural cycle were scattered in the stroma with aggregations around the glands (Figure 1a–c); however, the number of these leukocytes was notably reduced in the basalis. The leukocyte counts (CD45\(^+\), CD56\(^+\) and CD3\(^+\) cells) were low in the proliferative and early secretory phases, but were markedly increased in the late secretory and menstrual phases (Figure 2). Ki-67\(^+\) cells were present in the endometrial glands but more frequently expressed in the stroma (Figure 1d), and were generally more prevalent in the functionalis layer than the basalis. The Ki-67 antigen expression in the glands was raised
in the proliferative and early secretory phases, but declined in the late secretory and menstrual phases (Figure 3). In the stroma the Ki-67 expression remained relatively constant during the different phases of the natural cycle.

The total number of doubly labelled CD45<sup>+</sup>/Ki-67<sup>+</sup> cells showed a similar pattern of expression to their counterpart sections which were singly labelled for either CD45 or Ki-67 antigen, in terms of stromal scatter and aggregations around glands (Figures 4 and 1e,f). Glandular expression of Ki-67<sup>+</sup> in doubly labelled sections was similarly distributed compared to sections singly labelled for this antigen; however, the actual number of CD45<sup>+</sup> and Ki-67<sup>+</sup> cells was generally lower in the doubly labelled sections. Of the total CD45<sup>+</sup> cell count, only 13% were also positive for Ki-67 antigen.

**Trimegestone-treated endometrium**

The total number of endometrial specimens from trimegestone-treated women subjected to immunohistochemical analysis in this study was less than the total number of biopsies collected at the end of the 6 months of treatment. This was due to the fact that (i) two women declined to have a biopsy; (ii) 10 specimens from women who bled before the day of the biopsy were excluded; (iii) some samples were rather scanty; and (iv) other samples could not sustain the process of antigen retrieval. The numbers of evaluable sections therefore, were 95, 89, 98, 106 and 104 for CD45, CD56, CD3, Ki-67 and CD45/Ki-67 antigens respectively.

The mean count for CD45<sup>+</sup>, CD56<sup>+</sup> and CD3<sup>+</sup> cells in the endometrial biopsies obtained from trimegestone-treated women was not significantly different between the four dose groups (data not shown). However, endometrial sections from women who bled on the day of the biopsy contained higher numbers of CD45<sup>+</sup> (P = 0.043) and CD56 cells (P = 0.018) compared to sections from women who had not bled by then (unpaired t-test). There was no significant difference for CD3<sup>+</sup> cell count between these two groups.

To visualize the relationship between the findings for the treated groups, the four doses of trimegestone, and the four phases of the endometrium of the natural cycle we adopted the method of linear discriminant analysis. This utilizes multi-dimensional count data in two dimensions chosen to best distinguish between the groups.

Figure 5A shows the results for the CD45<sup>+</sup> cells. Three cell counts were made from each of the following fields: around the glands, in the stroma, and amongst intra-epithelial leukocytes (IEL). The two-dimensional plot captured 96% of the discriminant information. The profiles of counts in the treated groups were similar to those for the proliferative and early secretory phases of the natural cycle and were distinguished from endometrium of the late secretory and menstrual phases of the natural cycle mainly due to CD45<sup>+</sup> cell count around the glands.

The results for CD56<sup>+</sup> cells (Figure 5B) were similar to those for CD45<sup>+</sup> cells, with the treated groups resembling the proliferative and early secretory phases of the natural cycle. The discrimination occurred mainly along the first dimension for which a high value was associated with high stromal and IEL counts. The CD56<sup>+</sup> cell count around the glands did not help to distinguish the groups. CD3<sup>+</sup> cell count (Figure 5C) was similar but less clear, with only the menstrual phase well distinguished from the treated groups.

The Ki-67<sup>+</sup> cells were counted in the glands and the stroma (Figure 5D). In this case the proliferative and early secretory phases were distinguished from the treated groups by means of the Ki-67<sup>+</sup> cells in the glands.

Finally, the number of CD45<sup>+</sup>/Ki-67<sup>+</sup> doubly labelled cells (Figure 5E) produced six counts: CD45<sup>+</sup> cells around the glands, in the stroma and IEL cells, Ki-67<sup>+</sup> in the gland and stroma and the number of CD45<sup>+</sup>/Ki-67<sup>+</sup> cells in the stroma. The two-dimensional plot reproduced 85% of the discrimination and showed the treated groups to be similar to the menstrual phase of the natural cycle, with the late secretory phase of the natural cycle distinguished by a combination of higher Ki-67<sup>+</sup> cell counts in the glands and stroma, higher CD45<sup>+</sup>/Ki-67<sup>+</sup> cell count, and lower CD45<sup>+</sup> cell counts around the glands. The proliferative and early secretory phases were distinguished by the gland counts in Ki-67<sup>+</sup> and to a lesser extent in CD45<sup>+</sup> cells.

**Discussion**

Our results on the distribution and pattern of expression of endometrial CD45<sup>+</sup>, CD56<sup>+</sup> and CD3<sup>+</sup> cells in the natural cycle (Bulmer et al., 1991; Starkey et al., 1991; Klentzeris et al., 1992; Clark et al., 1996; Song et al., 1996) as well as the pattern of expression of Ki-67<sup>+</sup> cells (Shiozawa et al., 1996) are in agreement with the published literature. However, there is a lack of agreement in the literature as to the precise number of leukocytes and their subsets in any particular phase of endometrial development.

Our method of counting positively stained cells per unit area may be criticized for not making allowance for the size of the glands or for stromal oedema, where the number of stromal cells per unit area is reduced. However, the fact remains that the function of these lymphomyeloid cells is not dependent on the actual number of stromal cells, since there is about a 25% increase in the number of endometrial leukocytes in the late secretory phase of the natural cycle (Kamat and Isaacson, 1987).

Song et al. reported that a dose–response relationship existed between the administered progestogen (norethisterone and medroxyprogesterone acetate) and the endometrial stromal expression of the CD45<sup>+</sup> and CD3<sup>+</sup> cells, in which the response was significantly higher than the control (Song et al., 1996). Similar results were reported (Inoue et al., 1996). Our study did not show an effect of the dose of trimegestone on the expression of CD45<sup>+</sup>, CD56<sup>+</sup>, CD3<sup>+</sup> leukocytes or Ki-67<sup>+</sup> cells.

The expression of CD45<sup>+</sup>, CD56<sup>+</sup> and CD3<sup>+</sup> cells in the trimegestone-treated endometrium was similar to the proliferative and early secretory phases of the natural cycle. However, the expression of the proliferating marker Ki-67 and the proliferating doubly labelled leukocytes (CD45<sup>+</sup>/Ki-67<sup>+</sup>) was similar to the menstrual phase endometrium of the natural cycle. The reason that trimegestone had a different effect on leukocyte infiltration is not clear, but may well be related to
a different pattern of induction of progesterone-modulated chemotactic factors by stromal cells, such as RANTES protein (Hornung et al., 1998) or monocyte chemotactic protein-1 (MCP-1; Senturk et al., 1998). In addition, trimegestone may induce the expression of proliferation markers at an earlier phase of this HRT-treated endometrium compared with the natural cycle.

Our deployment of the linear discriminant analysis model was necessary to obtain an overview of the distribution of these antigens within the endometrial specimens, given the following variables: (i) the regional variability of the endometrium, (ii) a wider variability of cell counts within individual specimens, (iii) the separate description of positively stained cells for a particular antigen into glandular, periglandular and other stromal regions within an individual field, and (iv) the relatively large number of specimens used in this study, involving four doses of trimegestone and four phases of the natural cycle.

There was a higher number of endometrial leukocytes in women who were bleeding at the time of biopsy, which agrees with the proposed function of these cells in endometrial women who were bleeding at the time of biopsy, which agrees with the proposed function of these cells in endometrial women who were bleeding at the time of biopsy, which agrees with the proposed function of these cells in endometrial women who were bleeding at the time of biopsy, which agrees with the proposed function of these cells in endometrial women who were bleeding at the time of biopsy, which agrees with the proposed function of these cells in endometrial women who were bleeding at the time of biopsy, which agrees with the proposed function of these cells in endometrial women who were bleeding at the time of biopsy, which agrees with the proposed function of these cells in endometrial women who were bleeding at the time of biopsy, which agrees with the proposed function of these cells in endometrial women who 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