Endometrial expression of epithelial neutrophil-activating peptide-78 during the menstrual cycle or in progestin-only contraceptive users with breakthrough bleeding and the influence of doxycycline therapy

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BACKGROUND: Endometrial breakthrough bleeding is characterized by an inflammatory reaction and increased production of proinflammatory mediators, one of which may be epithelial neutrophil-activating peptide-78 (ENA-78), a chemokine with neutrophil-activating properties. METHODS AND RESULTS: We therefore investigated the endometrial expression of ENA-78 in Norplant users as progestin-only contraceptive with various bleeding patterns (n = 35) as compared with non-users with a normal menstrual cycle (n = 55). The endometrial stromal cells (ESCs) were the major site of ENA-78 expression with the highest levels found during the secretory phase. The expression of ENA-78 was increased in Norplant users with irregular bleeding as compared with those with regular cycles and amenorrhoea. The levels of ENA-78 detected in uterine washes and sera after the use of Norplant for 3–6 months (n = 25) increased compared with baseline (P < 0.05). These levels did not significantly change in Norplant users who received doxycycline (Dox) therapy (25 mg/twice daily for 6 months) when measured midway through or at the conclusion of study when compared with the baseline (n = 25). Treatments with medroxyprogesterone acetate (MPA) and tumour necrosis factor-α (TNF-α) (25 ng/ml), but not 17β-estradiol (E2) or E2 + MPA (10^-8 M), representing endometrium exposed to contraceptive and inflammatory conditions, respectively, increased the levels of ENA-78 production by ESCs, and this was reduced by co-treatments with Dox (25 μg/ml) (P < 0.05). CONCLUSIONS: The endometrial production of ENA-78 is altered in progestin-only contraceptive users experiencing breakthrough bleeding and is regulated by MPA and TNF-α in ESCs. Although Dox therapy did not alter uterine ENA-78 secretion, its suppression in ESCs suggests that Dox, acting site-specifically and through an anti-inflammatory mechanism, may influence the outcome of breakthrough bleeding in contraceptive users.

Key words: endometrium/ENA-78/doxycycline/ovarian steroids/uterine bleeding

Introduction

In recent years, doxycycline (Dox) and other tetracycline analogues have been found to be effective in medical management of disorders characterized by proinflammatory and protease activities (Brown et al., 2004; Preshaw et al., 2004; Salvi and Lang, 2005). The beneficial effects of these broad-spectrum antibiotics, including Dox, appear not to be due to their antimicrobial properties but rather due to the inhibition of proinflammatory cytokine production and protease activities (Brown et al., 2004; Lee et al., 2004; Preshaw et al., 2004; Siemonsma et al., 2004; Salvi and Lang, 2005).

An inflammatory response, characterized by increased production of proinflammatory mediators and proteases activities, has been considered to, at least in part, account for the molecular mechanism(s) responsible for endometrial tissue breakdown and breakthrough bleeding that often occur in contraceptive users (Jones et al., 2005; Rhoton-Vlasak et al., 2005; Smith and Critchley, 2005; Jabbour et al., 2006). Although breakthrough bleeding improves with increased duration of contraceptive
use, it is the major reason for a relatively high rate of non-compliance among contraceptive users. Treatment strategies for breakthrough bleeding in contraceptive users have been largely empiric and consisted of ethinyl estradiol, ibuprofen, oral contraceptives and levonorgestrel (Burkman et al., 2001; Cameron, 2001; Dunn et al., 2001; Munro, 2001). Previously, Dox therapy, considered to act through an antimicrobial mechanism, has been utilized for the management of pelvic inflammatory disease and premenstrual syndrome (Toto et al., 1988; Walters and Gibbs, 1990). A recent pilot study has also reported that short-term Dox therapy was as equally effective as mifepristone and mifepristone + ethinyl estradiol in reducing the number of days of uterine bleeding/spotting in progestin-only contraceptive users (Weisberg et al., 2006).

Additionally, in a mouse model of uterine bleeding, Dox treatment has been found to reduce protease activity (Kaitu’u et al., 2005), and in human endometrial cells under in vitro conditions, it has been shown to alter the production of proinflammatory cytokines and protease production (Li et al., 2006).

Epithelial neutrophil-activating peptide-78 (ENA-78)/CXCL-5 is a member of the CXC chemokines and was originally identified as a product of epithelial cells (Koch et al., 1994). ENA-78 is closely related to interleukin-8 (IL-8), another member of CXC chemokine family, and is considered to play a key role in neutrophil activation during the inflammatory reaction (Imaizumi et al., 1997). In addition to epithelial cells, the expression of ENA-78 has been identified in a number of other cell types and tissues including the endometrium (Koch et al., 1994; Imaizumi et al., 1997; Lukacs et al., 1998; King et al., 2002; Suzuki et al., 2002; Gear and Camerini, 2003; Imaizumi et al., 2004; McLoughlin et al., 2004). In the endometrium, the stromal cells are the major site of ENA-78 expression throughout the menstrual cycle, with the highest levels detected during the secretory phase (Nasu et al., 2001). The expression of ENA-78 in endometrial stromal cells (ESCs) has been reported to be regulated by progesterone, lipopolysaccharide, TNF-α and IL-1β (Nasu et al., 2001). Because of these specific properties, we hypothesized that endometrial expression of ENA-78 is altered during breakthrough uterine bleeding in contraceptive users. To test this hypothesis, we compared the expression of ENA-78 in endometrial biopsies from different phases of the menstrual cycle in (i) women with regular cycles (n = 55) and (ii) women experiencing breakthrough bleeding due to progestin-only contraceptive (n = 35). We also determined as a part of a placebo-controlled clinical trial whether Dox therapy, acting through an anti-inflammatory pathway, alters the uterine production of ENA-78 in women using progestin-only contraceptive (n = 50). Furthermore, in an in vitro model, consisting of endometrial glandular epithelial cells (GECs) and ESCs and a human endometrial surface epithelial cell line (HES), we investigated whether the production of ENA-78 was affected by Dox alone, or in combination with E₂, medroxyprogesterone acetate (MPA), E₂ + MPA or TNF-α, as these respectively represent endometrial environment exposed to contraceptives and inflammatory conditions.

Materials and methods

Study population

This study evaluated the expression of ENA-78 in uterine environment of three independent groups.

Group 1 consisted of women (n = 50) with regular menstrual cycles who were requesting permanent surgical sterilization (tubal ligation) at the University of Florida allied health departments during the period 1998–2000 (Chegini et al., 2002). The endometrial biopsies were collected from throughout the menstrual cycle with details provided in a previous study (Chegini et al., 2002).

Group 2 consisted of women (n = 570) who had Norplant insertions (levonorgestrel implants, Wyeth-Ayerst) at the University of Florida-affiliated Shands Hospital Clinics, or allied health departments during 1994–96 (Rhoton-Vlasak et al., 2005). These women were sent a questionnaire and were chosen if they responded and met the following inclusion criteria: (i) in one of the three subgroups (see below) based on bleeding pattern indicated on the questionnaire; (ii) ages 14–40 years (and if age <18, have had a living child); (iii) Norplant in place <5 years; (iv) weight <250 lb; and (v) negative urine pregnancy test, they were asked to provide an endometrial biopsy (Rhoton-Vlasak et al., 2005). On the basis of these criteria, endometrial biopsies (of sufficient size and quality) were obtained from women (n = 35) experiencing (i) regular cycles every 29–35 days (n = 13), (ii) amenorrhea, i.e. no bleeding for at least 2 months (n = 8), or (iii) continuous bleeding for 2–3 weeks at a time (n = 14). Endometrial biopsies were performed in patients from Groups 1 and 2 using a Pipelle (Prodimed, France).

Group 3 consisted of women (n = 50) chosen for an ongoing blinded, placebo-controlled clinical trial testing the efficacy of Dox in preventing uterine spot bleeding due to Norplant. The subjects were randomly divided into two groups with one group receiving Dox therapy, 25 mg twice daily for 6 months. These women were chosen if they met the study inclusion criteria, which included normal cycles, older than age 18, negative urine pregnancy test and negative vaginal infection. Norplant insertion was performed at Eastern Virginia Medical School-affiliated Clinics, or allied health departments. The uterine lavage (wash) and serum were collected at three time points: at baseline, at midway and at the conclusion of the 6-month clinical trial. The uterine washes were obtained in the following manner. A sterile transfer catheter was inserted into the uterine cavity. The uterine cavity was flushed with 1.0 ml of sterile saline in a to-and-fro manner for five times, and the fluid was then recovered by back pressure on the tuberculin syringe. A second wash with the same technique was then performed. The fluids were pooled and centrifuged, and aliquots were stored at −80°C until assayed. The uterine bleeding patterns were recorded throughout the duration of this study.

Institutional Review Board (IRB) approvals were obtained from both institutions before initiation of this study, and all patients gave informed consent.

Immunohistochemical studies

A proportion of the endometrial biopsies were fixed in neutral buffered formalin solution and embedded in paraffin. Tissue sections of 5 μm thick were prepared and, following haematoxylin and eosin staining, interpreted by a gynaecologic pathologist who was blinded to the patient’s bleeding pattern in Norplant users (Group 2). For immunohistochemical studies, tissue sections were treated with 0.01% Triton-X100 and 0.3% hyaluronidase before incubation with goat anti-human recombinant ENA-78 (R&D System, Minneapolis, MN, USA) at 5 μg/ml of IgG for 2–3 h at room temperature. The sections were exposed to biotinylated second antibody and avidin-conjugated horse-radish peroxidase (HRP; ABC Elite kit, Vector Biochemical, Burlingame, CA, USA), and the chromogenic reaction was detected with diaminobenzidine and
counterstained with haematoxylin. The omission of primary antibody or incubation of tissue sections with non-immune goat IgG instead of primary antibody at the same concentration served as controls. The staining intensity was determined using the HScore method. Briefly, following the assessment of control sections for non-specific staining, the cellular staining with very low, low, moderate or high intensity was recorded in a minimum of 400 cells, randomly selected from three fields of view for each specimen in a blinded fashion (Rhoton-Vlasak et al., 2005). HScore values were calculated with the equation 

\[ HScore = \sum P_i (i + 1) \]

where \( i \) = intensity of staining with a value of 0, 1, 2 or 3 (absent, weak, moderate or high intensity, respectively) and \( P_i \) = the percentage of stained cells varying from 0 to 100%.

**Endometrial cell isolation and culture**

The endometrial GECs and ESCs were isolated from portions of endometrial tissues as previously described (Chegini et al., 2002). The endometrial tissues were obtained from premenopausal women (\( n = 3 \)), ranging in age from 21 to 39 years, who were undergoing hysterectomy for medically indicated reasons (excluding endometrial cancer and endometriosis) at the University of Florida-affiliated Shands Hospital. These patients were not under any hormonal treatments at the time of surgery. The tissues were collected after obtaining approval from the University of Florida Institutional Review Board without requiring written informed consent. The endometrial cells were isolated and cultured in DMEM-Ham’s F-12 supplemented with 10% FBS. The purity of cell preparations was determined in freshly isolated cells and after first passage by immunostaining for cytokeratin (epithelial), vimentin (stromal) and a smooth muscle actin (smooth muscle), using their respective antibodies as previously described (Luo et al., 2004; Roberts et al., 2006). The cells were then cultured in 75-mm flasks in the presence of 10% FBS until reaching visible confluence. The human endometrial surface epithelial cell line (HES) was kindly provided by Dr D. Kniss (Ohio State University) and cultured as previously described (Luo et al., 2004).

All the materials for cell culturing were purchased from Fisher Scientific (Pittsburg, PA, USA) and Sigma Aldrich (St Louis, MO, USA), respectively. 17β-Estradiol (E2), MPA and Dox were purchased from Sigma, charcoal-stripped fetal calf serum (FCS) was purchased from Hyclone (Logan, UT, USA) and the recombinant TNF-α and an ELISA kit specific to human ENA-78 with a detection limit of 1 pg/ml were purchased from R&D System.

**The effect of Dox and ovarian steroids on ENA-78 expression**

The ESCs, GECs and HES were cultured at density of \( 2.5 \times 10^5 \) cells/well in 24-well dishes in the presence of 10% charcoal-stripped FCS and in phenol-red/serum-free conditions until reaching 70–80% confluence. The cells were maintained under a serum-free/phenol red-free conditions for 24 h and then treated with combinations of Dox (25 μg/ml) and E2, MPA or E2 + MPA at concentrations of \( 10^{-8} \) M added to phenol red-free medium containing 2% charcoal-stripped FCS. The cells were incubated for 24 h, and conditioned media were collected from treated and untreated controls and subjected to ELISA. The doses of Dox, E2, MPA and TNF-α and the duration of the incubation in the experiments of this study were based on our previous work with these cells (Chegini et al., 2002; Li et al., 2006; Roberts et al., 2006).

**The effect of TNF-α and Dox on ENA-78 expression**

The ESCs were cultured as described above and treated with TNF-α (25 ng/ml) in the presence or absence of Dox (25 μg/ml). The cells were incubated for 24 h, and conditioned media were collected from treated and untreated controls.

**ELISA**

The culture-conditioned media from the above experiments were centrifuged at 10 000 rpm for 15 min, and aliquots were made and stored at –80°C until they were subjected to ELISA. For ELISA, 150 μl of the uterine washes, sera or condition media was used to assay for ENA-78 levels essentially following the procedure described by the manufacturer (R&D System).

**Statistical analysis**

The results are presented as mean ± SEM. The HScore data were statistically analysed using non-parametric Kruskal–Wallis test. The in vitro experiments were performed using three independent cell cultures and performed in duplicate. The ELISA results were statistically analysed using Student’s t-test and ANOVA with Tukey test whenever appropriate. A probability level of \( P < 0.05 \) was considered significant.

**Results**

**Endometrial expression of ENA-78**

The endometrium throughout the menstrual cycle expressed ENA-78 with immunostaining predominantly localized in the stromal cell compartment (Figure 1A and B). ENA-78 immunostaining was also present to lesser extent in association with vascular endothelial/smooth muscle cells, with a limited staining of surface and GECs (Figure 1A and B). Semi-quantitative analysis (HScore) of the immunostaining intensity in the stromal cell compartment indicated that ENA-78 is expressed at a higher level in endometrial tissues from the early-mid secretory phase as compared with those from the proliferative phase of the menstrual cycle (Figure 2).

Immunostaining of ENA-78 in the endometrium of Norplant users experiencing regular cycles or amenorrhoea was lower than in the secretory phase, and in most instances similar to that observed in proliferative endometrium from non-users with normal menstrual cycles (Figure 1C). The immunostaining of ENA-78 in the endometrium of Norplant users with breakthrough bleeding was increased, predominantly in the stromal cell compartment, compared with that of Norplant users experiencing regular cycles, or non-users with normal menstrual cycles (Figure 1D). A strong immunostaining of ENA-78 was also detected in inflammatory cells often present among the endometrial epithelial (Figure 1E) and stromal cells (Figure 1F). Despite a considerable variation in ENA-78 immunostaining among different specimens, incubation of tissue sections with non-immune IgGs, serving as control, resulted in no, or reduced, staining (Figure 1G).

Semi-quantitative analyses (HScore) showed that endometrial ENA-78 staining intensity significantly was increased for Norplant users who experienced breakthrough bleeding (Group 2) as compared with that for non-users in the proliferative and secretory phases of the menstrual cycle [Group 1 (Figure 2; \( P < 0.05 \)]. The endometrial HScore of ENA-78 in Norplant users who experienced regular cycles or amenorrhoea was comparable to that in non-users from the proliferative phase of the menstrual cycle (Figure 2).
Uterine wash and serum levels of ENA-78 in Norplant users and the influence of Dox therapy

The levels of ENA-78 detected in uterine washes and sera at baseline ranged from 9 to 26 pg/ml and 900 to 1550 pg/ml, respectively. These levels were significantly increased in uterine washes, but not in sera, at midway (3 months) and at 6 months (i.e. the conclusion of the study) as compared with baseline levels (Figure 3A and B; \( P < 0.05 \)). However, ENA-78 levels of Norplant users were not significantly different in uterine washes or sera when compared with those of Norplant users who received Dox therapy; no difference was noted either at midway through the study or at 6 months when the study concluded (Figure 3).

The effect of Dox on ENA-78 production by endometrial cells

We used an in vitro model consisting of ESCs, GECs and HES cultured under defined conditions to determine the influence of Dox, ovarian steroids and a proinflammatory cytokine on
ENA-78 expression. As shown in Figure 4, only the ESCs produced ENA-78 (≈40 pg/ml) into their culture-conditioned media; GECs and HES produced very low to undetectable levels (data not shown). Therefore, the remaining experiments were performed using only ESCs. ENA-78 levels were significantly increased following treatment of ESCs with MPA, but not with E2, or E2 + MPA, as compared with untreated controls (Figure 4). In addition, treatment of ESCs with TNF-α (25 ng/ml) resulted in a significant increase in the production of ENA-78 (Figure 4). Dox (25 μg/ml) had no significant effect on ENA-78 production when compared with that of control, E2- or E2 + MPA-treated cells; however, Dox did significantly decrease MPA- and TNF-α-induced ENA-78 production (Figure 4).

Discussion
In this study, we showed that ENA-78, a CXC chemokine closely related to IL-8 with a key regulatory function in inflammatory reactions, is expressed in the endometrium throughout the menstrual cycle. The endometrial expression of ENA-78 was menstrual cycle-dependent with highest levels occurring during the secretory phase. Stromal cells were shown to be the major site of expression, and limited expression in surface and GECs was observed. These results are in agreement with a previous study in which ENA-78 expression was demonstrated in the endometrial tissues obtained from hysterectomy specimens (Nasu et al., 2001). We found that endometrial ENA-78 expression is altered in Norplant users experiencing breakthrough bleeding as compared with those who had regular cycles, or with non-users, suggesting a potential involvement of ENA-78 in events leading to focal endometrial tissue breakdown. Although Norplant as progestin-only contraceptive has been withdrawn from the market, in general, the mechanism of actions and patterns of breakthrough bleeding in Norplant users are similar to those seen with other contraceptive systems (Smith and Critchley, 2005).

In most contraceptive users, an endometrial inflammatory response, characterized by increased activities of proinflammatory mediators and proteases, is considered as a molecular mechanism(s) responsible for endometrial tissue breakdown and breakthrough bleeding (Jones et al., 2005; Rhoton-Vlasak et al., 2005; Smith and Critchley, 2005; Jabbour et al., 2006). As such, alteration in the endometrial expression of ENA-78, with key regulatory functions in neutrophil activation during the inflammatory reaction (Imaizumi et al., 1997), may be part of the above regulatory mechanism in Norplant users experiencing breakthrough bleeding. ENA-78 is also a member of ELR motif-positive CXC chemokines that regulate angiogenesis (Koch et al., 1994; Imaizumi et al., 1997; Lukacs et al., 1998; King et al., 2002; Suzuki et al., 2002; Gear and Camerini, 2003; Imaizumi et al., 2004; McLoughlin et al., 2004). Under such capacity and because of the expression in uterine vascular and inflammatory cells, ENA-78 can influence the endometrial vascular integrity and neutrophil populations.

Figure 2. Bar graph showing the mean ± SEM of immunostaining intensity (HScore) for ENA-78 in endometrial stromal cells of tissues from proliferative and secretory phases of the menstrual cycle and from Norplant users experiencing amenorrhoea (AM) or menorrhagia (M). The asterisks ** are significantly different from * (P < 0.05).

Figure 3. Bar graphs showing the mean ± SEM of ENA-78 levels in uterine washes (A) and sera (B) in Norplant users (Nor) or Norplant users receiving Dox therapy (Nor + Dox). Measurements were taken at baseline, midway through the study (midline) or at 6 months when the study concluded (final). The asterisks ** indicate statistically significant differences compared with * at baseline (P < 0.05).
that are altered during breakthrough bleeding in contraceptive users (Salamonsen et al., 2002; Jones et al., 2005; Smith and Critchley, 2005).

Elevated expression of ENA-78 during the secretory phase indicates that ovarian steroids, in particular progesterone, may regulate ENA-78. We found that MPA, but not E2, increased the production of ENA-78, whereas co-treatment with E2 + MPA reduced the production of ENA-78 when compared with that of stromal cells treated with MPA alone. The results suggest that increase in ENA-78 production in Norplant users could be due to local action of MPA, whereas the beneficial use of ethinyl estradiol and oral contraceptives as treatment strategy for controlling breakthrough bleeding in progestin-only contraceptive users (Burkman et al., 2001; Cameron, 2001; Dunn et al., 2001; Munro, 2001) may be due to reduction in the expression of inflammatory mediators such as ENA-78 production. Factors other than ovarian steroids may also regulate the endometrial expression of ENA-78 as demonstrated in ESCs following treatments with lipopolysaccharide, TNF-α and IL-1β (Nasu et al., 2001).

Dox has been studied for medical management of various disorders characterized by elevated levels of proinflammatory mediators and protease activity (Brown et al., 2004; Lee et al., 2004; Preshaw et al., 2004; Siemonsma et al., 2004; Salvi and Lang, 2005). Because the endometrial environment of women with breakthrough bleeding is similarly affected, as part of our ongoing clinical trial to test the efficacy of Dox therapy (25 mg twice daily for 6 months) on bleeding pattern, we used uterine washes and sera collected from women using Norplant as a contraceptive and found that the levels of ENA-78 in uterine washes, but not sera, significantly increased in Norplant users compared with baseline levels, and there were no alterations caused by Dox therapy. Because the endometrial surface and GECs produced low to undetectable levels of ENA-78, the most likely source of ENA-78 in the uterine washes could be the stromal cells or the vasculature. Alternatively, ENA-78 originates from serum, but the serum levels did not change during the course of the study. The results also suggest that the influence of Norplant (MPA) on ENA-78 is local with limited systemic activity, which is further supported by the effects of MPA on ENA-78 production by the ESCs. Earlier clinical studies have found Dox therapy, acting through its antimicrobial properties, useful for medical management of pelvic inflammatory disease and premenstrual syndrome (Toth et al., 1988; Walters and Gibbs, 1990). A recent pilot study has found that Dox at 100 mg twice daily for 5 days is equally effective as mifepristone and mifepristone + ethinyl estradiol in reducing the number of days of uterine bleeding/spotting in women using a progestin-only contraceptive (Weisberg et al., 2006).

The beneficial effect of Dox therapy in other disorders has been found to be not due to Dox’s antimicrobial activity but rather due to the inhibition of proinflammatory mediators and proteases production. Our study results indicated that despite the limited effect of Dox therapy on uterine ENA-78 secretion or baseline production by the ESCs, it significantly reduced MPA-induced ENA-78 production in cultured ESCs. We have recently reported that Dox alters the production of several other inflammatory and immune-related cytokines/chemokines in ESCs, GECs and HES without any cytotoxicity (Li et al., 2006). Unlike ENA-78, Dox treatment alters the production of other cytokines/chemokines, alone, or in combination with ovarian steroids as well as in cells co-treated with TNF-α, representing
an endometrial environment exposed to contraceptives and inflammatory conditions, respectively. Our study results with TNF-α induction of ENA-78 confirmed a previous study demonstrating the effect of TNF-α, IL-1β and endotoxin on ENA-78 production by ESCs (Nasu et al., 2001). Because Dox treatment reduced TNF-α-induced production of ENA-78 and other proinflammatory cytokines/chemokines (Li et al., 2006), Dox therapy may have beneficial effects not only for the management of breakthrough bleeding due to contraceptives but also in women affected by premenstrual syndrome (Toth et al., 1988).

In conclusion, the results indicate that endometrial expression of ENA-78 is altered in women experiencing breakthrough bleeding due to progestin-only contraceptives. Because the endometrial expression of ENA-78 was shown to be the target of progestin and Dox regulatory actions, the results support the hypothesis that Dox therapy may, independent of its antimicrobial property but through an anti-inflammatory pathway involving cytokine/chemokine production, alter the endometrial response to contraceptives. Considering ENA-78 functions as a key regulator of neutrophil activity, inhibition or reduction of its production may lead to the establishment of a favourable endometrial environment allowing for normal tissue repair in women experiencing breakthrough bleeding associated with contraceptive use.

Acknowledgement
This work was supported in part by NIH grants HD43175 and HD37432.

References