Endothelin (ET) and its mRNA are present in human endometrium. Its expression varies across the menstrual cycle, reaching maximal levels in the pre-menstrual phase. Human endometrial epithelial cells are the major in-vitro source of ET, and its release is affected by the stage of the menstrual cycle from which the endometrium is taken and by the cytokines transforming growth factor-β1 and interleukin-1α. The cyclical variation in both ET mRNA and protein expression across the normal menstrual cycle, and the differences observed in abnormal uterine bleeding (specifically reduced immunoreactivity in luminal and glandular epithelium in the endometrium of Norplant® users), are consistent with a role for ET in the control of menstrual bleeding. The role of epithelial cell ET is not yet understood. Whether this source of ET is important in endometrial regeneration and repair following menstruation or in vasoconstriction to cease menstrual bleeding remains to be determined.

Key words: endometrium/endothelin/menorrhagia/menstruation/Norplant®

Introduction
The mechanisms of normal menstrual bleeding remain largely unknown. The complex endometrial morphological changes linked to ovarian steroid production occurring during each menstrual cycle are consistent and have been documented in detail (Noyes et al., 1950). Observations in studies performed >40 years ago, where endometrium was transplanted into the anterior chamber of the eye in rhesus monkeys (Markee, 1940), form the basis of our knowledge regarding the microvascular changes that precede and accompany menstruation. Contraceptive steroids are associated with menstrual bleeding disturbances which vary according to the agent used. Menstrual disturbances are a major reason for the discontinuation of progestrone-only contraceptives (Odlind and Fraser, 1990). The mechanisms and factors responsible for the initiation of abnormal uterine bleeding associated with contraceptive steroid agents and other causes of abnormal uterine bleeding are not completely understood. There is, however, evidence that contraceptive steroids have specific cellular effects, particularly on blood vessel components (Johannisson, 1990).

Endothelin (ET), originally identified as a product of endothelial cells and having potent vasoconstrictor properties on vascular smooth muscle (Yanagisawa et al., 1988), is now also known to be a product of other cells, including certain types of epithelial cell (Macrae and Bloom, 1992; Kennedy et al., 1993; Masaki, 1993), and to have paracrine actions including mitogenesis of smooth muscle and fibroblasts. ET is present in normal human endometrium, and a potential paracrine role in the regulation of uterine blood flow has been suggested (Findlay et al., 1990; Economos et al., 1992; Cameron et al., 1993; Marsh et al., 1994). ET is inactivated by neutral endopeptidase (NEP) by non-specific cleavage of the mature ET molecule. In contrast to ET, NEP is localized predominantly in the stromal cells of human endometrium and is maximal during the early to mid-luteal phase (Casey et al., 1991; Imai et al., 1992; Head et al., 1993).
Studies of ET in normal human endometrium in our laboratory include the detection of mRNA, the immunolocalization of protein, in-vitro release from cultured human endometrial cells and in-situ hybridization. Endometrial biopsies from subjects with abnormal uterine bleeding have also been examined immunohistochemically for ET. These findings will be summarized and reviewed.

Detection of ET mRNA and immunolocalization of ET in normal endometrium

RNA was prepared from single human endometrial samples and a Northern analysis performed using a 1.2 kb cRNA ET-1 probe. ET-1 mRNA was present in increasing amounts towards the end of the menstrual cycle, with the greatest abundance detected in the secretory phase (Marsh et al., 1994). This is in agreement with data published by Economos et al. (1992).

ET immunoreactivity was detected in normal human endometrium (Salamonsen et al., 1992) using a polyclonal anti-human ET-1 (crossreactive with ET-2 and ET-3 but not big-ET). There was a cyclical variation of ET immunoreactivity in luminal and glandular epithelium, with maximal levels occurring in the mid- to late secretory phase, but decreasing in the menstrual phase with the loss of secretory characteristics in the epithelial cells. In contrast, stromal immunoreactivity remained relatively constant, although maximal ET immunoreactivity was observed in the menstrual phase.

When human endometrium, pre-incubated with the ionophore monensin (which blocks the passage of proteins through the Golgi apparatus) was subjected to immunohistochemistry for ET, immunoreactivity accumulated in glandular epithelium and endothelial cells but not stromal cells, suggesting that stromal immunoreactivity did not represent the production of the peptide by these cells (Salamonsen et al., 1992).

Studies using cultured human endometrial epithelial and stromal cells

To examine further the cellular source of endometrial ET, short-term cultures of human endometrial epithelial and stromal cells under serum-free (SF) conditions were used from individual or pooled endometrial specimens from either the proliferative (days 7–14) or secretory phase (days 16–24) of the menstrual cycle. Both epithelial and stromal cell cultures were ≥95% pure. ET was detected in the cytoplasm of cultured epithelial but not stromal cells using immunohistochemistry. ET was measured in the conditioned medium by a radioimmunoassay crossreactive with ET-2 (60%), ET-3 (70%) and big-ET (0.01%), having a mean sensitivity 1.4 ± 0.3 pg/ml (Marsh et al, 1994).

The total DNA content of each well was measured and the results expressed as mean ET per μg DNA (Marsh et al., 1994).

Endometrial epithelial but not stromal cells in primary culture released immunoreactive ET. Significantly more ET was released from separate cultures of epithelial cells than stromal cells prepared from four individual endometrial samples cultured in both fetal calf serum (FCS) and SF conditions (Marsh et al., 1994). Very little ET was released from any of the stromal cell cultures (Figure 1). This finding differs from the data of Economos et al. (1992), who have shown ET release by passaged stromal cells. While they showed that epithelial cells produced ~2.3 times more ET than stromal cells in a single experiment, studies of the regulation of ET production were performed on stromal cells after the first passage. Key differences between the Economos et al. (1992) study and ours, which may explain the
disparate findings, include the use of passaged stromal cells and longer term cultures compared with short-term culture; the use of FCS and subsequently FCS or no serum compared with the use of charcoal-stripped FCS followed by SF conditions; filtration through a 73 μm mesh in contrast to cell purification by sequential filtration through 45 and 10 μm filters; and a radioimmunoassay that was crossreactive with big-ET compared with one that was not (Marsh et al., 1994). Cultured human decidual cells from normal early pregnancy have been shown to release ET (Kubota et al., 1992), and stromal cells decidualize in long-term culture in the presence of progesterone (present in FCS). In a recent study, the release of small amounts of ET from passaged stromal cells was again shown (Kubota et al., 1995). In both the Economos et al. (1992) and Kubota et al. (1995) studies, the possibility that the stromal cells had decidualized in culture was not examined. Data suggesting that epithelial cells are the predominant source of ET released in culture are consistent with the immunohistochemical finding that ET is localized principally in epithelial cells in human endometrium (Cameron et al., 1992; Salamonsen et al., 1992).

When conditioned medium from human endometrial epithelial cells was subjected to reverse-phase high performance liquid chromatography (HPLC), the immunoreactive ET was found to elute at the same position as ET-1 standard (Marsh et al., 1994). In contrast, in extracts of whole human endometrium from both the proliferative and secretory phases, all three isoforms of ET were detectable by a reverse-phase HPLC analysis (Cameron et al., 1993). Cells other than epithelial cells may be responsible for the synthesis of the other isoforms found in intact tissue, or cofactors and conditions required for the production of these other forms from epithelial cells are not present in the culture system. The cell culture system does produce biologically active ET: HPLC fractions of human endometrial epithelial cell-conditioned medium containing ET immunoreactivity exhibited bioactivity equipotent to standard ET-1 in the induction of contraction of rat thoracic aortic rings denuded of endothelium, while fractions without immunoreactive ET showed no vasoactive response (Marsh et al., 1994).

<table>
<thead>
<tr>
<th>Treatment (concentration)</th>
<th>ET production (mean % control ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS (10%)</td>
<td>339 ± 159 (^{b})</td>
</tr>
<tr>
<td>TGF-β1 (2 ng/ml)</td>
<td>157 ± 11 (^{b})</td>
</tr>
<tr>
<td>TGF-β1 (5 ng/ml)</td>
<td>155 ± 5 (^{b})</td>
</tr>
<tr>
<td>TGF-β1 (10 ng/ml)</td>
<td>169 ± 14 (^{b})</td>
</tr>
<tr>
<td>IL-1α (1 IU/ml)</td>
<td>118 ± 35</td>
</tr>
<tr>
<td>IL-1α (10 IU/ml)</td>
<td>118 ± 35</td>
</tr>
<tr>
<td>IL-1α (100 IU/ml)</td>
<td>142 ± 10 (^{b})</td>
</tr>
</tbody>
</table>

*Data expressed as mean percentage of control (serum free; 100%) ± SEM.

**Significant stimulation of ET release (P < 0.05) above control.

Basal ET release by human endometrial epithelial cells was affected by the stage of the menstrual cycle from which the cells were derived. Proliferative epithelial cells consistently produced significantly more ET than secretory cells, and cells from both the proliferative and secretory phases cultured in FCS produced more ET compared with those cells cultured in SF medium. FCS stimulated ET release from epithelial cells above control levels in both proliferative (214%) and secretory (301%) phase endometrium (Marsh et al., 1994). These data are not consistent with those from Northern blot analyses and immunohistochemical studies, where higher levels of ET-1 mRNA and more immunoreactive ET in epithelial cells are found during the secretory phase. It is likely that regulatory influences on ET mRNA and peptide synthesis by endometrium in vivo are not available to purified cultured endometrial epithelial cells, or that the in-vitro release of ET may have been altered by the previous in-vivo hormonal environment.

The release of ET by epithelial cells was significantly stimulated above control levels by FCS in a dose-dependent manner (Marsh et al., 1994). The stimulation of ET release above control levels was also produced by transforming growth factor (TGF)-β1 (2–10 ng/ml) and interleukin (IL)-1α (10 and 100 IU/ml) (Table I). Neither stimulated ET release to the degree observed with FCS. Therefore serum factors other than TGF-β1 must exist which either stimulate the synthesis or release of ET.
or inhibit its metabolism. Alternatively, in-vivo exposure of the endometrium to TGF-β1 may be altering the subsequent responsiveness to TGF-β1 in culture (Marsh et al., 1994). There was no change in ET release in response to any of epidermal growth factor, oxytocin, vasopressin thrombin, angiotensin II or phorbol myristate acetate factors which have been shown in other cell types to stimulate ET release at the concentrations tested (Figure 2). The regulation of ET production by TGF-β1 and IL-1α has been shown in other cell types (Brown et al., 1990, 1991; Yoshizumi et al., 1990; Economos et al., 1992; Masaki, 1993), and their mRNA has been detected in human endometrium (Yoshizumi et al., 1990; Tabibzadeh, 1992). Thus both TGF-β1 and IL-1α are potentially important paracrine regulatory factors in ET production from endometrial epithelial cells.

Abnormal uterine bleeding
Endometrial biopsies from subjects with abnormal uterine bleeding have been examined immunohistochemically for ET and the results compared with findings in the normal menstrual cycle. Two groups of subjects with abnormal uterine bleeding were compared: those with bleeding associated with the use of slow-release levonorgestrel (Norplant) and another group with menorrhagia.

Slow-release levonorgestrel, a long-acting subdermally implanted progestogen, is an effective widely used contraceptive. There is a high rate of discontinuation of use (20–30%) because of unacceptable menstrual bleeding disturbances (Odlind and Fraser, 1990). In endometrial tissue taken from these women during the first year of contraceptive use the overall immunostaining pattern for ET resembled that of the normal proliferative phase. In general, ET immunostaining was low in glandular and luminal epithelium, with the strongest staining in the stroma (Figure 3, black bars). The ET immunostaining pattern in endometrium was similar in all patients treated with Norplant, irrespective of the number of menstrual bleeding days observed or the number of days since the last bleeding episode. There was no correlation with the immunostaining pattern, the duration of implant use or serum oestradiol or progesterone concentrations. Neither was there a correlation between the duration of implant use, the number of menstrual bleeding days and steroid concentrations (Marsh et al., 1995). NEP (assessed by immunohistochemistry in the endometrial biopsies of subjects using Norplant) was localized principally in endometrial stromal cells, but positive staining was also found in the glandular epithelium. Compared with the normal menstrual cycle, glandular immunostaining was increased while stromal immunostaining was similar in intensity to the proliferative phase. The increased NEP immunoreactivity in glandular epithelium may rep-
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Figure 4. Schematic representation of human endometrium and proposed actions of epithelial cell endothelin in the peri-menstrual period.

represent a local increase in enzyme activity, potentially increasing the metabolism of ET. This finding may provide an explanation for the reduced ET immunoreactivity in glandular epithelium in the endometrial biopsies of users of Norplant (Marsh et al., 1995).

Endometrial biopsies from subjects with menorrhagia, documented by a measured monthly menstrual blood loss >80 ml, also showed substantially different ET immunoreactivities to that of the normal menstrual cycle. ET immunoreactivity was less intense in glandular and luminal epithelium than that observed at the same stage of the normal menstrual cycle, and less intense than stromal immunostaining which was of similar intensity to the normal menstrual cycle (M.M.Marsh, J.K.Findlay and L.A.Salamonsen, unpublished observations).

Discussion

The cyclical variation in both endometrial ET mRNA and protein expression across the normal menstrual cycle, and the reduced immunoreactivity in luminal and glandular epithelium observed in endometrium of women with abnormal uterine bleeding, are consistent with a role for ET in the control of menstrual bleeding. Human endometrial cells have been shown to release ET in vitro, and this has been identified as ET-1 by reverse-phase HPLC and shown to be bioactive. The release of ET by human endometrial epithelial cells was stimulated by FCS, TGF-β1, and IL-1α, and was altered by the stage of the cycle from which the endometrium was derived. While cultured endometrial cells release ~10-fold greater amounts of ET than cultured human endometrial epithelial cells (Brown et al., 1991), human endometrial stromal cells in culture release 2- to 10-fold lower quantities of ET than cultured endometrial epithelial cells (Economos et al., 1992; Marsh et al., 1994; Kubota et al., 1995).

ET receptors and their subtypes have been identified in normal human endometrium by autoradiography (Davenport et al., 1991; Bacon et al., 1995), and mRNA for both ET_A and ET_B receptor subtypes have been detected in human endometrial RNA from across the menstrual cycle using polymerase chain reaction techniques and Northern blot analyses (O’Reilly et al., 1992; Pekonen et al., 1994; Kubota et al., 1995). The identification of ET receptor subtypes in normal human endometrium provides the mechanism for ET to act on the endometrium. To date, in human endometrium these receptors have been localized in glandular epithelium and blood vessels, although the specific function of the ET receptor subtypes in the human endometrium has yet to be defined.

The role of epithelial cell ET in the endometrium is not known. We propose that this ET has an important action in endometrial repair and regeneration following menstruation. In addition, ET may act to constrict basal arterioles following menstruation to halt menstrual bleeding (Figure 4).
It does not seem likely that epithelial cell ET could be available to spiral arteries to induce constriction before the onset of menstruation. The lack of a correlation of ET immunostaining in Norplant with the number of bleeding days suggests that ET is not likely to be a key factor in the observed increased uterine bleeding. Rather, we postulate that the reduced epithelial cell ET results in impaired endometrial regeneration and repair, leading to a fragile endometrium and/or inadequate vasoconstriction of the basal blood vessels, to cease bleeding once this has been initiated. The precise roles of ET in human endometrium, and the in vivo regulators of ET production in the endometrium remain to be determined.

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References


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