The role of tissue factor in regulating endometrial haemostasis: implications for progestin-only contraception

C.J. Lockwood, R. Runic, L. Wan, G. Krikun, R. Demopolous and F. Schatz

Departments of Obstetrics and Gynecology and Pathology, New York University School of Medicine, New York, NY 10016, USA

To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, New York University School of Medicine, New York, NY 10016, USA.
E-mail: charles.lockwood@med.nyu.edu

Abnormal uterine bleeding accounts for the unacceptably high discontinuation rate of progestin-only contraceptives. Previously, we found that in-vivo and in-vitro decidualization of human endometrial stromal cells was associated with elevated concentrations of tissue factor (TF), the primary initiator of haemostasis. Moreover, enhanced TF expression required progesterone receptor (PR) and epidermal growth factor receptor (EGFR) mediation. In the current study, endometrial biopsies were sampled from bleeding (BL) and non-bleeding (NBL) sites under camera-directed hysteroscopic guidance after Depo-provera injections. When compared with control biopsies, immunohistochemical examination revealed that 3 months of Depo-provera contraception reduced TF concentrations at the BL sites. However, there were ample EGFR and PR concentrations at BL and NBL sites. Moreover, there was a trend towards the appearance of pathologically enlarged blood vessels at the BL sites. The use of Western blotting revealed that after 3 months of Depo-provera, concentrations of both PR_B and PR_A isoforms were lower at BL versus NBL sites with decreased PR_A concentrations attaining statistical significance. Separate sampling of endometrial BL and NBL sites as shown here for Depo-provera contraception could prove particularly useful in identifying local factors that determine the onset of bleeding during the more protracted time-course of Norplant® contraception.

Key words: abnormal uterine bleeding/fragile vessels/haemostasis/progesterone-only contraception/tissue factor

Introduction

Previously, we found enhanced concentrations of tissue factor (TF) in decidualized stromal cells of luteal phase and pregnant endometria (Lockwood et al., 1993a). Tissue factor is a 46 kDa cell membrane-bound glycoprotein consisting of a hydrophylic extracellular domain, a membrane-spanning hydrophobic domain, and a cytoplasmic tail (Bach, 1988; Nemerson, 1988). Upon exposure to blood, the extracellular domain of TF binds to circulating factor VII or its active form Vila (Bach et al., 1986; Hagen et al., 1986). By directly or indirectly activating factor X, the TF-factor Vila complex converts prothrombin to thrombin. Cleavage of fibrinogen by thrombin leads to haemostasis-promoting fibrin deposition. Although TF is not normally expressed by cells in contact with circulating blood, its expression by perivascular cells forms a protective ‘haemostatic envelope’ (Carmeliet and Collen, 1988).

In human endometrial stromal cell monolayers isolated from specimens of cycling endometrium, progestins were found to enhance concentrations of TF mRNA and protein (Lockwood et al., 1993b). Despite a lack of response to oestradiol alone, oestradiol plus progestin further augmented TF mRNA and protein expression (Lockwood et al.,...
Tissue factor and endometrial haemostasis

1993b). Similar differential responses to oestradiol and progestin were reported for such classical decidualization markers as prolactin (PRL), insulin-like growth factor binding protein (IGFBP)-1 and plasminogen activator inhibitor (PAI)-1 (Huang et al., 1987; Irwin et al., 1989; Giudice et al., 1991; Schatz and Lockwood, 1993). These effects mimic differential ovarian steroid actions in vivo in which oestradiol primes human endometrial stromal cells for the decidualizing effects of progesterone by elevating progesterone receptor (PR) concentrations (Eckert and Katzenellenbogen, 1981). Thus, cultured human endometrial stromal cells are a good decidualization model. Moreover, we observed that TF mRNA and protein concentrations were elevated for at least three weeks during progestin-induced in-vitro decidualization of human endometrial stromal cells (Lockwood et al., 1993b). These in-vitro effects simulate the long-term up-regulation of TF that occurs in association with decidualization in vivo (Lockwood et al., 1993b, 1994).

The absence of oestrogen or progestin response elements from the TF promoter taken together with reports that steroid–epidermal growth factor (EGF) interactions control growth and differentiation in the mouse uterus (Tamooka et al., 1986; DiAugustine et al., 1988; Nelson KG, 1991) and in cultured human endometrial stromal cells (Irwin et al., 1991), prompted us to evaluate progestin–epidermal growth factor (EGF) effects on TF expression in the latter. Induction of TF mRNA and protein required exogenous progestin and EGF receptor (EGFR) agonists such as EGF or transforming growth factor-α (TGF-α). By contrast, TGF-β and interleukin-1β, which do not activate the EGFR, failed to enhance TF mRNA and protein concentrations in cultured human endometrial stromal cells whether added alone or with progestin. Moreover, EGFR concentrations were elevated during decidualization in vitro as determined by Western blotting (Lockwood et al., 2000).

Endometrial tissue factor expression during progestin-only contraception

Previous study of Norplant-2® effects

Progestin-only contraception is among the most effective and safest methods of birth control available. Norplant-2 consists of two subdermally implanted Silastic brand rods (Dow Corning, Midland, MI, USA), which provide 5 years of 99% contraceptive efficacy by releasing levonorgestrel. Prolonged uterine bleeding as well as irregular breakthrough bleeding and spotting between ‘cycles’ are the most common patient complaints and the major reasons for discontinuation (Gu et al., 1994) of this efficacious mode of contraception.

In a retrospective study (Runic et al., 1997), we evaluated concentrations of TF protein by immunohistochemistry and mRNA by in-situ hybridization in 24 endometrial specimens obtained by Pipelle biopsy following Norplant-2 contraception. Compared with TF expression in secretary phase controls, Norplant-2 treatment elicited respective reductions in endometrial TF mRNA and protein concentrations of about 200% and 300% after 1–6 months and 6–12 months. These intervals correspond to the periods of heaviest bleeding. After 12 months of Norplant-2 contraception, a modest rebound in TF mRNA and protein concentrations occurred commensurate with reduced abnormal uterine bleeding. However, microscopic examination revealed the presence of pathologically enlarged blood vessels, suggestive of increased vascular fragility, in endometrial biopsies obtained after 1 year on Norplant-2 (Runic et al., 1997). Thus, continuation of abnormal uterine bleeding appeared to result primarily from impaired vascular integrity.

Current study of Depo-provera effects

Background and rationale

The prototypical injectable progestin-only contraceptive, Depo-provera (Sigma, St. Louis, MO, USA), lacks the multi-year effectiveness of Norplant. Moreover, abnormal uterine bleeding is generally more severe during the initial 3 months of Depo-provera than with Norplant administration. In the case of Depo-provera, the initial period of heavy bleeding is generally followed by amenorrhoea, which may be advantageous to specific subgroups of women. In addition, unlike implantable contraceptives which must be removed surgically by qualified medical personnel, discontinuation of Depo-provera is automatic.

The current study sought to evaluate effects of

145
Depo-provera administration on the haemostatic and angiogenic status of the endometrium. Thus, concentrations of PR and EGFR, which are required to maintain elevated TF concentrations in decidualized stromal cells of normal endometrium (Lockwood et al., 2000) were measured in the endometrium following Depo-provera injections. The effects of long-term progesterin-only contraception on PR expression have been actively investigated. Such therapy was expected to down-regulate the oestrogen receptor (ER), thereby suppressing PR concentrations and eliciting a local hypoprogesteronal endometrial milieu. However, it was reported that Norplant-exposed endometria displayed the expected reduced ER immunostaining, whereas immunohistochemical PR concentrations were paradoxically up-regulated (Critchley et al., 1993). The subsequent demonstration of PR\(_A\) and PR\(_B\) isoforms with the former antagonizing the effects of the latter (Tung et al., 1993) prompted evaluation of the effects of long-term progesterin-only contraception on endometrial PR isoform status. In the absence of a PR\(_A\) antibody, PR\(_A\) concentrations were deduced by subtracting immunostaining intensity observed with an antibody against PR\(_B\) from that obtained with an antibody that recognizes both PR\(_A\) and PR\(_B\) isoforms, i.e. total PR (Critchley et al., 1998; Wang et al., 1998). The current study sought to improve upon this subjective assessment by measuring PR concentrations in Depo-provera-derived endometria through the use of Western Blotting, which separates the isoforms based on size differences, in conjunction with immunohistochemical staining for total PR and PR\(_B\). Depo-provera effects on endometrial EGFR concentrations were also assessed by immunohistochemistry. In view of the association between the appearance of enlarged vessels in the endometrium following 3–12 months of Norplant treatment (Runic et al., 1997), the morphological appearance of endometrial vessels were also evaluated in the current study. Biopsies were sampled separately from bleeding (BL) and non-bleeding (NBL) sites under camera-directed hysteroscopic guidance in order to relate the status of the various measured endpoints to the local endometrial haemostatic milieu.

### Materials and methods

#### Tissues

After obtaining written informed consent and approval by the Institutional Board of Research Associates of NYU Medical Center and Bellevue Hospital, endometrial specimens were obtained from normally cycling women before (control group), and after 3 months of Depo-provera contraception. Control samples were obtained from either the proliferative or secretory phases of the cycle by pipelle biopsy (Unimar, Willon, CT, USA). Norplant-derived specimens were obtained from six patients using a 5 mm operative hysteroscope (Karl Storz Endoscopy-America Inc, Culver City, CA, USA) under video camera guidance from both BL (Figure 1A) and NBL (Figure 1B) sites. For each patient studied, at least one pre-Depo-provera control biopsy and biopsies from both BL and NBL sites after 3 months of Depo-provera treatment were obtained. The biopsies were divided into fragments, which were fixed in 4% paraformaldehyde, or embedded in paraffin, or immediately frozen in liquid nitrogen prior to storing at -80°C. Specimens with obvious myometrial components were excluded from immunohistochemical and morphological studies.

#### Immunohistochemistry

Tissue sections (5 μm thick) were deparaffinized, quenched, microwave-heated and incubated overnight at 4°C with either: (i) 1:80 dilution of monoclonal anti-progesterone receptor (PR) antibody (Novocastra Laboratories, Newcastle, UK); (ii) 10 mg/ml of mouse anti-PR\(_B\) isoform antibody (clone KC 146) from Dr G.Greene (University of Chicago, Chicago, IL, USA); (iii) 1:500 dilution of rabbit anti-TF from Dr Y.Nemerson (Mount Sinai School of Medicine, New York, NY, USA); or (iv) 1:20 dilution of monoclonal anti-EGFR antibody (Zymed, San Francisco, CA, USA). Negative controls were incubated with the same dilution of mouse or rabbit anti-IgG antibody. Washed sections were treated with anti-mouse or anti-rabbit-peroxidase conjugate and the colour was developed with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Haematoxylin was used as a counterstain.
Hysteroscopic biopsy of bleeding and non-bleeding sites following 3 months of Depo-provera contraception. (a) Bleeding site. The hysteroscopic biopsy forceps demarcates the area of bleeding. As noted in Results, bleeding sites were sampled from the fundal part of the uterus close to the tubal ostium (TO). (b) Non-bleeding site.

Figure 1. Hysteroscopic biopsy of bleeding and non-bleeding sites following 3 months of Depo-provera contraception. (a) Bleeding site. The hysteroscopic biopsy forceps demarcates the area of bleeding. As noted in Results, bleeding sites were sampled from the fundal part of the uterus close to the tubal ostium (TO). (b) Non-bleeding site.

Quantification of microscopic measurements
Intensity of immunohistochemical staining and measurements of vessel density, lumen width, estimated by red blood cell diameter, as well as endothelial cell width and vascular smooth muscle thickness were quantitated by two blinded independent observers (R.R. and R.D.). Previously, we described a semi-quantitative scoring system to ranging from none, weak, moderate, and strong to assess relative intensity of immunohistochemical staining in endometrial specimens obtained during Norplant contraception (Runic et al., 1997).

Western blotting
Frozen tissues were Dounce-homogenized in 4 vols of ice-cold RIP buffer (25 mmol/l Tris, 150 mmol/l NaCl, 10 mmol/l EGTA, 2 mmol/l EDTA, 0.5% Nonidet P-40, pH 7.6) containing a protease inhibitor cocktail and centrifuged at 800 g for 5 min at 4°C. The supernatants were centrifuged at 100 000 g for 1 h at 4°C; the resulting supernatant yielded the cytosolic fraction for PR (Imai et al.,

Figure 2. Immunohistochemical staining for the epidermal growth factor receptor (EGFR) and progesterone receptor (PR) in endometrial bleeding and non-bleeding sites after 3 months of Depo-provera. The results shown are typical of five individual patients. (A) EGFR staining in a day 16 control specimen; ×100. (B, D) EGFR staining in a BL site after 3 months of Depo-provera injections; Original magnifications (B) ×100, (D) ×400. (C, E) EGFR staining in a NBL site after 3 months of Depo-provera injections; (C) ×100; (E) ×400. (F) PR staining in a BL site after 3 months of Depo-provera injections; ×400. (G) PR staining in a non-bleeding site after 3 months of Depo-provera injections; ×400. Scale bar = 50 μm.
Supernatants were concentrated by an Ultrafree-4 centrifugal filter with a 30 kDa threshold. After resolution by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) (60 μg protein/lane) under denaturing conditions, Western blotting was performed with monoclonal anti-PR (1:200 dilution) (Neomarkers, Fremont, CA, USA). Detection was by ECL Western blotting protocols (Amersham, Arlington Heights, IL, USA). Densitometry and printing were performed with the Kodak Digital Science Camera System; statistics were performed using the Sigma Stat/Sigma Plot (St Louis, MO, USA).

Results

Figure 1 demonstrates that BL sites can be readily distinguished from NBL sites under camera-directed hysteroscopic guidance. The position of the biopsy forceps indicates that each site can be sampled separately. Note that in this study, care was taken to sample BL sites from the fundal part of the uterus close to the tubal ostium (TO). This was done in order to avoid sampling the lower uterine segment near the cervical canal where trauma resulting from dilating the cervix could cause bleeding artifacts.

Figure 2A shows a pre-Depo-provera control endometrial specimen obtained at day 16 of the menstrual cycle after immunohistochemical staining for the EGFR. Particularly strong immunohistochemical staining is seen in the stromal cells around the blood vessels and glands. However, even stronger immunohistochemical staining for the EGFR is evident in the stromal cells around the blood vessels (arrowhead) and glands (g) in both the BL (B, D) and NBL (C, E) sites of a specimen obtained after 3 months of Depo-provera contraception.

Figure 2 also indicates that the stromal cells at both the BL (F) and NBL (G) sites of the Depo-provera-derived endometrial specimen stained prominently for the presence of the PR. The antibody used recognizes both known PR isoforms: PRB (116 kDa) and PRA (84 kDa). To determine the relative abundance of the two isoforms, Western blotting was carried out on endometrial extracts from BL and NBL sites after 3 months of Depo-provera injections. Figure 3 compares the abundance of the two isoforms at BL and NBL sites by relative densitometry after resolution by size on SDS–PAGE. The NBL sites contained higher concentrations of PR, PRB and PRA than seen at BL sites. However, only for PR did the higher concentrations at NBL compared with BL sites attain statistical significance.

When compared with the pre-Depo-provera control endometrium, the immunohistochemical staining shown in Figure 4 reveals that Depo-provera treatment preferentially lowered TF concentrations in the stromal cells (s) of endometrial BL sites. Morphological examination of five of these specimens indicates that 3 months of Depo-provera contraception resulted in enlarged blood vessels (arrowheads) at the BL sites versus NBL sites. These consisted of an average 10% increase in lumen diameter and 25% decrease in smooth muscle wall thickness. The failure of these measurements to attain statistical significance may relate to the relatively short exposure time and small number of specimens available.

Discussion

The results presented in the current report extend our previous study of haemostatic-related effects of Norplant contraception to include the injectable progestin-only contraceptive Depo-provera. In contrast with Norplant administration, injectable formulations require a much greater degree of patient
compliance, i.e. injections every 3 months in the case of Depo-provera. Moreover, abnormal uterine bleeding associated with the use of injectable contraceptives are generally more severe than with implantable contraceptives. Against these disadvantages, administration of injectable contraceptives is easily discontinued. In studying the effects of Depo-provera, the current report expands our previous study in two important ways. Unlike the latter in which endometrial biopsies were obtained non-selectively, camera-directed hysteroscopic guidance was now used to individually sample endometrial BL and NBL sites following 3 months of Depo-provera contraception. This approach enabled us to determine whether these sites displayed differences in the expression of endpoints involved in the regulation of haemostasis. As with our Norplant study, such markers included immunohistochemical staining for TF and microscopic examination of vascular morphology. Moreover, in light of our findings that enhanced TF expression in endometrial stromal cells during decidualization is mediated by both the PR and EGFR (Lockwood et al., 2000), the current study also measured both receptors in endometrial BL and NBL sites.

In endometrial biopsies obtained after 3 months of Depo-provera injections, immunohistochemical concentrations of EGFR, PR and TF were readily demonstrable in BL and NBL sites. Consistent with our observations of progestin-enhanced EGFR concentrations during in-vitro decidualization (Lockwood et al., 1993a, 2000), EGFR concentrations were higher at both sites after 3 months of exposure to the synthetic progestin Depo-provera compared with the luteal phase control. Although concentrations of total PR as well as the specific PR_B and PR_A isoforms were lower at BL than at NBL sites, only the reduction in PR_A concentrations proved statistically significant. Predicting the effects of these changes in PR concentrations on TF expression is equivocal. Thus, lowered PR_A concentrations may effectively enhance PR activity since in some tissues PR_A has been shown to antagonize the actions of PR_B (Tung et al., 1993; Graham and Clarke, 1997). However, there is
suggesive evidence that the PR_A isoform may mediate progesterone effects on stromal cells of luteal phase human endometrium (Wang et al., 1998). Compared with luteal phase control, TF staining was weaker at BL sites following Depo-provera treatment, and enlarged vessels were detected at the BL sites. The latter changes were accelerated compared with our previously reported effects of Norplant in which abnormally enlarged vessels were first evident after 6 months to 1 year (Runic et al., 1997). Thus, as with our previous Norplant study, the occurrence of BL may reflect a combination of reduced TF-mediated haemostasis and increased vessel fragility.

Separate sampling of endometrial BL and NBL sites by camera-directed hysteroscopic guidance, as shown here for Depo-provera contraception, could prove particularly useful in identifying local factors that regulate the onset of bleeding following Norplant administration given the prolonged time-course involved in this form of contraception. In this regard, the recent realization that beyond its classical role of initiating haemostasis, TF is also a potent mediator of angiogenesis (Zhang et al., 1994; Carmeliet and Collen, 1998) could prove instructive. Thus, TF expression is enhanced for less than a week during the normal menstrual cycles, whereas the much more prolonged expression of TF that occurs during progestin-only contraception may lead to aberrant angiogenesis and produce distended, fragile vessels. Investigating the potential relationship between TF expression in Norplant-derived endometrial BL and NBL sites and the expression of such transcription factors as EGR-1 and members of the promoter-specific transcription family (Sp1 and Sp3) may prove fruitful in view of our recent finding that these factors are involved in TF expression during decidualization of human endometrial stromal cells (Krikun et al., 2000). In addition to TF, there are several classical regulators of angiogenesis whose expression in Norplant-derived endometrial BL and NBL sites is also worth investigating (see recent reviews by Smith, 1998; Healy et al., 1998). Chief among these are fibroblast growth factor and vascular endothelial growth factor together with their cognate receptors (reviewed in Hyder and Stancel, 1999). Comparative analysis of endometrial BL and NBL sites promises to be a valuable modality with which to unravel mechanisms regulating bleeding during progestin-only contraception.

Acknowledgements

This work was supported in part by grants from the National Institutes of Health 5 R01 HD33937-05 and from the General Clinical Research Center M01 RR00096.

References


Tissue factor and endometrial haemostasis


