In-vivo angiogenesis and progestogens

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BACKGROUND: Progestogens are used clinically for contraception, to control excessive menstrual bleeding, and to prevent estrogen-induced endometrial hyperplasia. A significant problem with progestogen-only methods of contraception is the induction of breakthrough bleeding. METHODS: The effects of different progestogens on angiogenesis were examined using two approaches. The mouse sponge angiogenesis assay employed direct delivery of the dose ranges achieved therapeutically. The angiogenic response to long-term intrauterine levonorgestrel exposure, compared with unexposed premenopausal endometrium, was also studied. RESULTS: In the mouse sponge assay, norethisterone and medroxyprogesterone acetate stimulated angiogenesis at all doses, but was dose-dependent for levonorgestrel and nomegestrol. Levonorgestrel stimulated angiogenesis in the dose range 100 pmol/l to 10 nmol/l, but not at higher doses. In contrast, nomegestrol acetate stimulated angiogenesis at high, but not low, doses. Expression of acidic and basic fibroblast growth factors, thymidine phosphorylase, vascular endothelial growth factor and adrenomedullin were unaltered in levonorgestrel-exposed endometrium compared with premenopausal controls. Vascular density was increased but endothelial proliferation reduced in levonorgestrel-exposed endometrium. CONCLUSIONS: This is the first report of the direct effects of a wide range of doses of different progestogens on angiogenesis; results suggest that vascular targeting may be an effective strategy to deal with progestogen-induced abnormal bleeding.

Key words: angiogenesis/endometrium/endothelial proliferation/progestogens/vascular density

Introduction

Progestogens are used clinically for contraception, to control excessive menstrual bleeding, and to reduce the risk of estrogen-induced endometrial neoplasia (Irvine and Cameron, 1999; d’Arcangues, 2000; Rees, 2000; Riphagen, 2000).

Two main groups of progestogens are used for these indications: 19-norprogestogen and 17-hydroxyprogesterone derivatives (e.g. medroxyprogesterone acetate). The 19-norprogestogens are further divided into those derived from progesterone (e.g. nomegestrol acetate) or testosterone (e.g. levonorgestrel, norethisterone).

A significant problem with progestogen-only methods of contraception is the endometrial bleeding disturbances that these methods induce (d’Arcangues, 2000). However, much higher doses of progestogens are often used to treat abnormal bleeding patterns (Milsom et al., 1991; Irvine and Cameron 1999). Intrauterine delivery of progestogens such as levonorgestrel, which produces high endometrial steroid levels, induces amenorrhea in most women by one year, and has proven to be a successful treatment for menorrhagia (Milsom et al., 1991). That the dose of progestogen used may influence bleeding patterns has also been found with hormone replacement therapy regimes, with higher doses of progestogen giving better amenorrhoea rates with continuous combined therapy (Archer et al., 1999; Heikkinen et al., 2000).

Aberrant endometrial angiogenesis has been implicated in abnormal bleeding (Gargett and Rogers, 2001), and the effects of progestogens have been examined in response to oral and depot delivery. Abnormalities in endometrial blood vessels have been noted with oral progestogens used for contraception (Hourihan et al., 1990). Low-dose depot progestogens can cause an increase in microvascular density, but with reduced endothelial proliferation (Rogers et al., 1993; Goodger et al., 1994). The increased vascular density has been found as soon as 3 weeks after insertion of a subdermal levonorgestrel contraceptive implant (Hickey et al., 1996, 1999). In contrast, high-dose oral progestogens reduce microvascular density (Song et al., 1995). Furthermore, different types of progestogens may have varied effects (Wahab et al., 2000).

Data regarding the effects of progestogens on angiogenesis are limited. Medroxyprogesterone acetate has been reported as having direct anti-angiogenic activity in a corneal assay (Yamamoto et al., 1994), and this was abolished by the use of mifepristone. Using the chick chorioallantoic membrane
were dewaxed and rehydrated and incubated in a trough of distilled water at 37°C for 10 min and in 200 ml of phosphate-buffered saline (PBSA: 0.13 mol/l NaCl, 0.002 mol/l KCl, 0.01 mol/l Na3HPO4, 0.002 mol/l KH2PO4) containing 25 mg protease type 24 (Sigma, Poole, Dorset, UK) at 37°C for a further 10 min. Slides were then incubated in 0.1% trypsin for 20 min at 37°C and washed twice in PBS.

The sections were immunostained with an antibody against von Willebrand factor, diluted 1:400 for 1 h at room temperature. The sections were rinsed in PBSA and incubated with a biotinylated goat anti-rabbit antibody (Dako) diluted 1:100 for 30 min at room temperature. The vessels were visualized with 3,3’-diaminobenzidine (DAB) (Dako) and counterstained with haematoxylin.

**Assessment of sponge vascularity**

The vascularity of the explanted sponges was determined by Chalkley counting (Fox et al., 1995). Vascular density was determined using a 25-point Chalkley eyepiece graticule (Graticules Ltd, Edenbridge, Kent, UK) at ×250 magnification (the graticule covers an area of 0.155 mm² at this magnification) in three areas selected at random. The graticule was rotated in the eyepiece to where the maximum number of vessels was overlaid by graticule dots. Individual density was then obtained by taking the mean of three graticule counts.

**Preparation of tissue**

Formalin-fixed, wax-embedded specimens of endometrium were obtained from archival files of hysterectomy and endometrial biopsy samples collected between 1993 and 1999. Tissue collection was performed in accordance with the requirements of the Central Oxford Regional Ethics Committee (COREC C2519). Thirty premenopausal hysterectomy samples were obtained from women (aged 30–45 years) undergoing hysterectomy for a subjective complaint of menorrhagia. No pelvic pathology was seen at operation. This was confirmed by a subsequent histological examination by an independent histopathologist. All patients had a history of regular 26- to 30-day menstrual cycles, and had not used oral or intrauterine contraception, nor taken any hormones for at least 6 months prior to surgery. The stage of the menstrual cycle at which the tissue was obtained was determined from the patients’ menstrual history and endometrial histology (Wells, 1996): 10 menstrual, 10 follicular and 10 luteal. Endometrial biopsy samples were obtained from 30 premenopausal women who had been using an intruterine device containing levonorgestrel (Mirena®; Leiras Oy, Finland) for contraceptive purposes (n = 2), or a complaint of menorrhagia (n = 28). The women were aged 38–45 years, and had had the device in situ for 3 years, at which time the aspiration biopsy was performed and a replacement device inserted. Biopsy was undertaken since the levonorgestrel intrauterine device had at that time a licence for 3 years use in the UK. The women had been amenorrhoeic for 30 months or more, though some had experienced some light spotting. None of the women had received any other hormonal treatment.

**Immunohistochemistry**

Immunohistochemistry was essentially as described previously (Hague et al., 2000). In brief, all sections were dewaxed using Citroclear (HS Supplies, Aylesbury, UK), rehydrated sequentially in absolute, 95%, 70%, 20% ethanol, distilled water, and finally rinsed in Tris-buffered saline (TBS) (pH 7.6) prior to staining.

**aFGF and bFGF immunohistochemical staining**

Immunohistochemical staining for aFGF was undertaken using the streptavidin–biotin–peroxidase (ABC) method using the Vectastain ABC kit for peroxidase (rabbit IgG) (Vector Laboratories, Burlingame, USA) according to the manufacturer’s protocol with rabbit anti-bovine aFGF polyclonal antibody (Sigma, UK) at a dilution of 1:200
in TBS/20% swine serum (SS). Sections were washed twice in TBS, and then developed with DAB. Immunohistochemistry of bFGF was performed using the same method, with rabbit anti-bovine bFGF polyclonal antibody (Sigma) diluted to 84 ng/ml in TBS.

**TP immunohistochemical staining**

Immunohistochemical staining for TP was carried out using the streptavidin–biotin–alkaline phosphatase (ABC) method. Before application of the primary antibody, the sections were incubated with 20% normal rabbit serum (NRS) to block non-specific protein binding sites. Primary antibody, PGF44C (ICRF, UK), was added to the slides for 30 min. The slides were then washed twice in TBS and incubated for a further 30 min with biotinylated rabbit anti-mouse immunoglobulin at 1:50 dilution. The sections were then washed again in TBS and incubated with streptavidin 1:200 (Dako) for 30 min. Chromogen development was performed using a ‘New Fuschin’ Substrate System (Dako) according to the manufacturer’s instructions by incubation for 5–10 min. Slides were counterstained with haematoxylin, and mounted with Apathy’s mounting medium (British Drug Houses, Dorset, UK).

**VEGF immunohistochemical staining**

Immunohistochemical staining for VEGF was carried out using the streptavidin–biotin–alkaline phosphatase (ABC) method, as described previously (Zhang et al., 1998). The ‘New Fuschin’ Substrate System was used to visualize the sections. In the negative controls the primary antibodies were replaced with the same subtype of mouse immunoglobulin (Sigma) at the same concentration.

**Adrenomedullin immunohistochemical staining**

Immunohistochemistry was carried out as described previously (Zhao et al., 1998). Briefly, slides were incubated with 5% goat serum (Dako) to reduce non-specific background staining, followed by 1:800 anti-adrenomedullin (Peninsula Laboratories, Liverpool, UK). A second biotinylated swine anti-rabbit antibody (Dako) at a dilution of 1:400 was then applied for 30 min, after which the slides were incubated with streptavidin 1:200 (Dako) for 30 min, the final colour being developed with the ‘New Fuschin’ Substrate System (Dako).

**Ki-67/CD34 double staining**

Immunohistochemical staining for CD34 was performed using the streptavidin–biotin–alkaline phosphatase (ABC) method. Throughout the protocol, all antibody dilutions and washes were performed in TBS, with all incubations being performed at room temperature. Antigen retrieval was performed by means of pressure-cooking in 1.6 litres 0.01 mol/l sodium citrate buffer (pH 6.0) for 90 s, followed by a 30 min rinse in distilled water and 5 min in TBS. Before application of the primary antibody the sections were blocked in 10% normal human serum. Sections were incubated at room temperature for 30 min with the primary CD34 antibody (Qbend 10; Novacastra, Newcastle upon Tyne, UK), diluted 1:25. Again, the sections were quenched by application of 0.3% hydrogen peroxide, diluted in distilled water. The sections were washed, and incubated in horse serum, after which the primary Ki-67 antibody (Bio Genex, San Ramon, CA, USA) at a dilution of 1:10 was added for 30 min. The sections were washed and incubated with the secondary biotinylated ABC antibody for 30 min, and the final colour was developed with DAB substrate (Sigma).

**Scoring of immunohistochemical staining**

The staining for aFGF, bFGF, TP, VEGF and ADM was scored on a scale of 1–3+, with 3+ indicating a strong positive result. The stroma, epithelium and endothelium were scored separately. Sections were examined by two reviewers (S.H. and M.R.). The final score credited to each sample was agreed upon by the two examiners.

**Determination of vascular density and endothelial cell proliferative indices**

Vascular density was determined by Chalkley counting in three areas selected at random (Fox et al., 1995). Vessels were counted using a 25-point Chalkley eyepiece graticule at ×250 magnification. The graticule was rotated in the eyepiece to where the maximal number of vessels were overlaid by graticule dots. Individual density was then obtained by taking the mean of three graticule counts.

The endothelial cell proliferative index was determined at ×400 magnification. The index was calculated as the percentage of all Ki-67 positively stained endothelial nuclei that also had concomitant positive cytoplasmic staining in CD34-positive cells.

**Statistical analysis**

Analysis of microvascular density, endothelial cell proliferation index and angiogenic factor immunostaining was carried out using the non-parametric Mann–Whitney U-test.

**Results**

The angiogenic activity of the progestogens levonorgestrel, medroxyprogesterone acetate, nomegestrol acetate and norethindrone was tested using the in-vivo mouse subcutaneous sponge angiogenesis assay (Figures 1–3; Table I).

**Levonorgestrel**

While levonorgestrel significantly stimulated angiogenesis at concentrations of 100 pmol/l, and 1 and 10 nmol/l (P < 0.004), higher concentrations (100 nmol/l and 1 μmol/l) had no stimulatory effect.

**Medroxyprogesterone acetate**

All concentrations of medroxyprogesterone acetate studied significantly increased angiogenesis over the physiological saline control (P < 0.004).

**Nomegestrol acetate**

Concentrations of 10 nmol/l and 100 pmol/l nomegestrol acetate significantly stimulated angiogenesis (P = 0.001 and P = 0.002 respectively), but showed no stimulatory effect at a concentration of 1 pmol/l.

**Norethisterone**

Norethisterone significantly increased angiogenesis at concentrations of 10 fmol/l, 1 pmol/l, 1, 10 and 100 nmol/l, and 1 μmol/l (P < 0.01).
Expression of angiogenic factors in levonorgestrel-exposed endometrium

aFGF
Negative or very weak expression was detected in control endometrium, localizing predominantly to the glandular epithelium, stroma and endothelium of the blood vessels. No changes in expression levels were observed through the menstrual cycle. In the levonorgestrel-exposed samples, expression of aFGF was again very weak, localizing to the epithelium, stroma and the endothelium of the blood vessels. Statistical comparison of the staining levels between control and levonorgestrel samples showed no significant differences in expression levels in the epithelium \( P = \text{NS} (0.73) \), stroma \( P = \text{NS} (0.72) \) or endothelium of the blood vessels \( P = \text{NS} (0.951) \).

bFGF
Expression was observed in the normal endometrial sections examined, localized predominantly to the glandular epithelium and, to a reduced level, in the stroma and endothelium. Expression was increased in the proliferative phase, and reduced in the secretory phase of the menstrual cycle. Again, the expression of bFGF in the levonorgestrel exposed samples was localized to the epithelium, stroma and endothelium of the blood vessels. However, statistical analysis revealed no significant differences in the level of staining between the control and levonorgestrel-exposed samples [epithelium, \( P = \text{NS} (0.766) \); stroma, \( P = \text{NS} (0.875) \); endothelium, \( P = \text{NS} (0.12) \)].

Thymidine phosphorylase
Immunostaining was present throughout the menstrual cycle in control endometrium, being most intense in the glandular epithelium in the late secretory and menstrual phases. Staining of the endometrial stroma and endothelium was observed at a lower level. A low level of TP expression was observed in the endometrium of the levonorgestrel-exposed samples. Expression was localized to the epithelium and the stroma, but no expression was detected in the endothelial cells. A statistical comparison showed there to be no significant difference in expression levels between the control and levonorgestrel groups [epithelium, \( P = \text{NS} (0.575) \); stroma, \( P = \text{NS} (0.799) \); endothelium, \( P = \text{NS} (0.626) \)].

VEGF
In the control samples, expression was present throughout the menstrual cycle, being detected principally in the glandular epithelial cells and at a reduced level in the stroma. The highest level of staining was seen in the menstrual, early proliferative and late secretory phases of the cycle. Expression in the endothelium of blood vessels was also observed. Expression of VEGF was observed in the epithelium, stroma and blood vessels of the levonorgestrel samples examined, at approximately the same level as the controls. A statistical comparison showed there was no significant difference in expression levels of the control and levonorgestrel groups [epithelium, \( P = \text{NS} (0.246) \); stroma, \( P = \text{NS} (0.461) \); endothelium, \( P = \text{NS} (0.19) \)].

ADM
The stromal, epithelial, endothelium and macrophages of the normal premenopausal endometrium stained positive for ADM. There was no cycle-specific change of expression. The glandular epithelium, stroma and blood vessels stained weakly positive for the presence of ADM in the levonorgestrel-exposed samples. Statistical analysis revealed no significant differences in levels of expression between the levonorgestrel and control samples [epithelium, \( P = \text{NS} (0.292) \); stroma, \( P = \text{NS} (0.965) \); endothelium, \( P = \text{NS} (0.542) \)].

Mean vascular density and endothelial proliferation index
The mean vascular density of the levonorgestrel-exposed samples was 4.7 (range 3–7), whilst that for the control samples was 2.5 (range 1–4). Statistical comparison showed that levonorgestrel samples had a significantly higher vascular density than controls \( (P = 0.001) \). (Figure 3A). The endothelial cell proliferation index was determined by counting the number of CD34 positively stained cells and the percentage of them that were positive for Ki-67 (Figure 4). The mean endothelial cell proliferation rate for the levonorgestrel-exposed endometrial samples was 3.78% (range 1–9%), and that for control samples was 10.7% (range 6.7–17.5%). A statistical comparison showed the endothelial proliferation index in the levonorgestrel group to be significantly lower \( (P < 0.0001) \) (Figure 3B).
Figure 2. Assessment of microvascular density in the mouse subcutaneous sponge assay, after immunostaining for von Willebrand factor. (A) Levonorgestrel; (B) medroxyprogesterone acetate; (C) nomegestrol acetate; (D) norethisterone. Values are mean ± SE (n = 3). *, significant increase in vascular density when compared with control.

Figure 3. (A) Comparison of endometrial vascular density between the levonorgestrel-treated and control samples. (B) Comparison of endothelial proliferation index between levonorgestrel-treated and control endometrium. *, P < 0.001; **, P < 0.0001.

Discussion
The angiogenic activity of the two main groups of progestogens, namely 17-hydroxyprogesterone (medroxyprogesterone acetate) and 19-norprogestogen (norethisterone, levonorgestrel and nomegestrol acetate) were assessed using two in-vivo approaches. The mouse subcutaneous sponge angiogenesis assay examined acute exposure using a span of progestogen concentrations which included those achieved therapeutically, and chronic exposure with the levonorgestrel-exposed endometrium. Data regarding levels of progestogens achieved therapeutically were obtained either from publications, from the manufacturers’ data sheets or manufacturers’ file data. Endometrial data are limited to oral and intrauterine levonorgestrel delivery. The levels of levonorgestrel achieved in the endometrium with intrauterine delivery are much higher than with oral administration, being in the region of 808 ng/g tissue compared with 3.5 ng/g tissue (Nilsson et al., 1982). For other gestagens and delivery systems, data are confined principally to serum levels. A subdermal levonorgestrel-releasing device (Norplant®, Leiras Oy), which has now been withdrawn in some countries, achieved a serum concentration of ~1 pmol/l (Croxatto, 1993). For nomegestrol acetate, the C_{max} after a single injection of 5 mg is 34.6 pmol/l (data on file obtained from Theramex Laboratories, Monaco). Medroxyprogesterone acetate administered as a depot injection (Depo Provera® 150 mg/ml achieves 6.8 ± 0.8 nmol/l medroxyprogesterone acetate, 2 weeks after injection; data sheet, Pharmacia and Upjohn Ltd). Norethisterone, administered orally at a dose of 1 mg, achieved a C_{max} of 20 nmol/l (data from Janssen Cilag Ltd, Saunderton, Bucks, UK), and 0.5 mg norethisterone combined with 1 mg 17β-estradiol gave a C_{max} of 1 nmol/l.
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Figure 4. Double immunohistochemical staining for Ki-67 and CD34. The Ki-67 antigen was stained using a streptavidin–biotin–peroxidase (ABC) method (brown), and the CD 34 antigen was stained using an alkaline phosphatase method (red). ▲, proliferating endothelial cell. Original magnification, ×400. Scale bar = 50 μm.

Table 1. Comparison of vascular density between progestogen-stimulated mouse sponges and control sponges injected with physiological saline

<table>
<thead>
<tr>
<th>Progestogen</th>
<th>Concentration</th>
<th>Mean vascular density</th>
<th>Significant increase in vascular density over control samples</th>
<th>P</th>
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M = mol/l; NS = not significant.

(Kliovance® data sheet; Novo Nordisk Pharmaceuticals Ltd, Crawley, West Sussex, UK).

In the mouse sponge assay, norethisterone and medroxyprogesterone acetate stimulated angiogenesis at all doses, but for levonorgestrel and nomegestrol acetate the stimulatory effect was dose-dependent. Levonorgestrel stimulated angiogenesis in the dose range 100 pmol/l to 10 nmol/l, but had no effect at higher doses. The dose range reflects that
achieved with oral and intrauterine administration. In contrast, nomegestrol acetate stimulated angiogenesis at high, but not low, doses.

Chronic exposure of the endometrium to intrauterine levonorgestrel was associated with increased vascular density but not increased endothelial proliferation. This pattern has also been found in long-term subdermal levonorgestrel users (Rogers et al., 1993; Goodger et al., 1994). It is of interest that high doses of levonorgestrel did not significantly stimulate angiogenesis in the mouse sponge assay. Furthermore, the increased vascular density may be accounted for by a reduced rate of regression of the blood vessels relative to the remainder of the endometrial tissue (Rogers et al., 1993).

Expression of αFGF, bFGF, TP, VEGF and ADM was unaffected in levonorgestrel-exposed tissue, though data are limited. The expression of VEGF has previously been examined in relation to levonorgestrel exposure delivered either into the uterus (Charnock-Jones et al., 2000) or subdermally in an implant (Lau et al., 1999). Intrauterine treatment with levonorgestrel resulted in strong glandular epithelial staining and intense staining of decidualized stromal cells; similar results were reported by others (Lau et al., 1999). The differences between the results of the present study and those reported elsewhere (Lau et al., 1999; Charnock-Jones et al., 2000) may be explained by the different antibodies employed. Although it has been shown previously, using a monoclonal antibody validated for immunohistochemical analysis by transfection experiments, that some antibodies do not definitively recognize VEGF, the monoclonal antibody used in this study did show such recognition (Zhang et al., 1998).

The mechanisms by which progestogens affect angiogenesis are uncertain, since the evidence that endothelial cells—including those in the endometrium—contain steroid receptors is conflicting, and some studies have shown there to be a lack of receptors (Crichtley et al., 1993; Perrot-Applanat et al., 1994). Recently, the presence of estrogen receptor beta (but not alpha) has been described in the endometrial endometrium (Crichtley et al., 2001; Lecce et al., 2001). Furthermore, the presence of progesterone receptors has been reported in human decidual endothelial cells during pregnancy (Wang et al., 1992), and more recently in normal and subdermal levonorgestrel users (Rodriguez-Manzaneque et al., 2000). Experiments performed in vitro on isolated human endometrial endothelial cells revealed that progesterone exposure leads to suppression of endothelial cell proliferation (Rodriguez-Manzaneque et al., 2000). Examination of estrogen and progesterone receptors in endometrium exposed to intrauterine levonorgestrel showed a marked reduction in both the glandular epithelium and stroma, but changes in the endothelium were not documented (Hurskainen et al., 2000; Jones and Crichtley, 2000).

Levonorgestrel may act through the androgen receptor, to which it binds (Kloosterboer et al., 1988). Androgen receptors have been described in endothelium in bone marrow (Abu et al., 1997) and also in levonorgestrel-exposed endometrium (Jones and Crichtley, 2000).

In conclusion, it appears that progestogens are able to induce angiogenesis at doses encountered using systemic delivery. However, at higher doses, for example in the range of those achieved with intrauterine administration of levonorgestrel, angiogenesis appears to be reduced. This observation is relevant to the amenorrhoea induced by long-term exposure to intrauterine levonorgestrel and its use to reduce endometrial hyperplasia provoked by the administration of estrogen (Riphagen, 2000), and to revert such changes (Perino et al., 1987). This is the first report of the direct effects of a wide range of doses of different progestogens on angiogenesis, and supports the idea that anti-angiogenics would be an effective strategy to deal with the abnormal bleeding induced by progestogen-only contraceptives.

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References


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