Inhibin and activin subunits are differentially expressed in endometrial cells and leukocytes during the menstrual cycle, in early pregnancy and in women using progestin-only contraception

R.L. Jones1,5, L.A. Salamonsen1, H.O.D. Critchley2, P.A.W. Rogers3, B. Affandi4 and J.K. Findlay1

1 Prince Henry’s Institute of Medical Research, P.O. Box 5152, Clayton, Victoria 3168, Australia, 2 Department of Obstetrics and Gynaecology, University of Edinburgh, Centre for Reproductive Biology, Edinburgh, EH3 9EW, UK, 3 Monash University Dept. of Obstetrics and Gynaecology, Clayton, Victoria 3168, Australia, and 4 Human Reproduction Study Group, Department of Obstetrics and Gynaecology, University of Indonesia, Klinik Raden Salah, Jalan Raden Salah 49, Jakarta, 10330, Indonesia

Inhibins and activins are dimeric hormones which share common subunits and which have diverse endocrine and paracrine roles in regulating reproductive function. Endometrial expression of inhibin α, βA and βB subunits was examined by immunohistochemistry and in-situ hybridization, across the menstrual cycle and in early pregnancy. All three subunits were found to be expressed in endometrium, primarily by glandular epithelium in the early stages of the cycle. Following the onset of decidualization, expression of α, βA and βB subunits was up-regulated in decidualized stromal cells. A marked down-regulation of α subunit was detected in glandular epithelium, whilst expression of βA and βB subunits was maintained. This pattern was consistent in decidua from early pregnancy and additionally in endometrium from women using progestin-only contraceptives, either subdermal implants (Norplant®) or levonorgestrel-releasing intrauterine systems (LNG-IUS). Immunostaining was also observed for both βA and βB subunits in subpopulations of endometrial leukocytes, identified to be distinct subsets of macrophages, neutrophils and mast cells. Potential paracrine roles for activins may be envisaged in facilitating tissue remodelling during decidualization, in tissue repair following menstruation, and additionally in modulating premenstrual inflammatory events.

Key words: activin/decidualization/endometrium/inhibin.leukocyte

Introduction

Inhibins and activins are closely related dimeric glycoproteins belonging to the transforming growth factor-β (TGF-β) super-family. Inhibins are heterodimers of a unique α subunit, with one of two β subunits, βA or βB, producing inhibin A (α;βA) or inhibin B (α;βB). Dimerization of β subunits alone produces activins: the most common detected forms being activins A (βA;βA), AB (βA;βB) and B (βB;βB).

Inhibins and activins were identified originally as factors acting antagonistically in the endocrine regulation of pituitary FSH production (Ling et al., 1986; Vale et al., 1986). Recent descriptions of the expression of inhibins and activins in numerous cell types and tissues indicates diverse functions, particularly as paracrine modulators of reproductive function (Mather et al., 1992; Knight, 1996; Lockwood et al., 1998), including ovarian function (Findlay, 1993) and gonadal tumorigenesis (Matzuk et al., 1992; Burger et al., 1998). Activin A has been attributed with functions in modulating cellular proliferation, apoptosis, wound healing, erythropoiesis, steroidogenesis and embryogenesis (reviewed by Mather et al., 1992; Findlay, 1993; Woodruff, 1998). Maternal serum concentrations of inhibins and activins are elevated during pregnancy, with concentrations being positively correlated with gestation and reaching a maximum at term (Woodruff et al., 1997; Qu and Thomas, 1998). The major source is believed to be the placenta (Qu and Thomas, 1995), although other components of the maternal–fetal unit, including the fetal membranes and decidua, are potential contributors (Petraglia et al., 1990; Keelan et al., 1998).

The endometrial expression of inhibin subunits has been examined previously, but significant controversy remains in the literature. Although one group (Otani et al., 1998) failed to detect the presence of the α subunit, a recent report from our group described the localization of the αN/αC subunit in non-pregnant endometrium (Leung et al., 1998). This is an important issue, as the availability of α subunit is likely to determine which active dimers (inhibins or activins) are preferentially formed, with subsequent implications regarding functions within the uterus. Complementary results describe βA expression by non-pregnant endometrium (Leung et al., 1998; Otani et al., 1998), while βB, but little βA, protein was detected in tissue collected from early pregnancy (Petraglia et al., 1990). Recently, mRNA for all three subunits was detected by reverse transcription–polymerase chain reaction (RT–PCR) from cultured endometrial epithelial and stromal cells (Petraglia et al., 1998), but this gives no information about cellular distribution in intact tissue or fluctuations in protein levels with the prevailing steroid environment. Inconsistencies in the literature regarding expression of inhibin subunits means it is impossible to ascribe functions for the
active dimers in the endometrium. By detailed measurement of $\alpha$, $\beta$A and $\beta$B subunit mRNA and protein expression in the same tissues, we aim to produce a definitive description of inhibin subunit expression patterns throughout the menstrual cycle and in early pregnancy.

This paper provides evidence for the expression of both the mRNA and protein for all three inhibin/activin subunits by human endometrium, with distinct elevations observed during the decidualization process in early pregnancy. Further, the regulatory role of progestin in vivo was investigated by the study of inhibin/activin subunit expression in women receiving exogenous levonorgestrel, delivered either subdermally via s.c. implants (Norplant®) or directly into the uterine cavity, via a levonorgestrel-releasing intrauterine system (Lng-IUS; Mirena, Leiras Oy, Finland). These results suggest that activins in particular play an important contributory role in modulating endometrial function, especially during the decidualization of the endometrium in the peri-implantation period and early pregnancy.

Materials and methods

Tissue collection and patient details
All tissue collections were approved by the appropriate Institutional Human Ethics Committees. Written informed consent was obtained from all women participating in the study.

Non-pregnant and pregnant endometrium
Endometrial biopsies ($n = 50$) were collected by dilation and curettage during minor gynaecological investigations from fertile women reporting regular menstrual cycles and with no history of endometrial pathology. The stage of the menstrual cycle was confirmed by histological dating according to previously described criteria (Noyes, 1950); thereafter samples were allocated to one of five groups: menstrual (days 1–4, $n = 9$), proliferative (days 5–13, $n = 12$), and early (days 14–17, $n = 8$), mid (days 18–24, $n = 8$) and late secretory (days 25–28, $n = 13$). In addition, first trimester decidua parietalis ($n = 5$) was collected by curettage prior to suction termination of pregnancy (8–10 weeks amenorrhoea). Absence of fetal trophoblast invasion was confirmed by cytokeratin immunohistochemistry and examination of sections stained with haematoxylin and eosin.

Levonorgestrel-releasing intrauterine system (Lng-IUS)
Endometrial biopsies were collected by Pipelle suction curette (Laboratoire CCD, Paris, France) following exposure to high local concentrations of synthetic progestin via a levonorgestrel-releasing intrauterine system. Women ($n = 10$) were recruited for contraceptive purposes or for the treatment of menorrhagia. This study was of longitudinal design with each patient acting as her own control (Critchley et al., 1998). An endometrial biopsy was thus taken prior to insertion of the device, either in the proliferative ($n = 5$) or secretory phase ($n = 5$) of the menstrual cycle, and at 1, 3, 6 and 12 months post-insertion. Endometrial cyclic activity is completely abolished following insertion of the device, thus post-insertional endometrial samples at each of the time points were examined independent of stage of cycle.

Norplant
Endometrial samples from women exposed to systemic levonorgestrel via subdermal implants (Norplant) were obtained from the Klinik Raden Salah in Jakarta, Indonesia. Between 3 weeks and 12 months after Norplant insertion, an endometrial sample was collected by Pipelle suction curette. These samples were divided into three groups according to their morphology: proliferative ($n = 5$), progesterone modified ($n = 8$) or shedding ($n = 4$) (Vincent et al., 1999).

Tissue treatment
Endometrial biopsies were immersion fixed overnight (17 ± 1 h) in 10% buffered formalin at 4°C, before thorough washing with Tris-buffered saline (TBS, pH 7.6) and routine histological paraffin embedding. Tissue sections (5 µm) were cut for immunohistochemical analysis.

Immunohistochemistry
Localization of $\alpha$, $\beta$A and $\beta$B subunits was determined using affinity purified polyclonal antibodies generated in rabbits against the individual inhibin subunits (anti-porcine inhibin $\alpha$2–26; anti-porcine inhibin $\beta$A41–113; anti-human inhibin $\beta$B80–112), whose specificity has previously been validated (Vaughan et al., 1989; Gandolfi et al., 1995; Yamashita et al., 1999). A number of positive control tissues, with established expression patterns for each of the three subunits, were included for further confirmation of the specificity of the immunostaining, including human term placenta (Petraglia, 1997) and amniochorion (Keelan et al., 1998).

Tissue sections were dewaxed in histosol and rehydrated through decreasing grades of ethanol. A microwave antigen retrieval step was required for all inhibin subunits, involving boiling tissue sections for 3×5 min at full power (1000 W) in 0.01 mol/l sodium citrate (pH 6.0), followed by 20 min cooling. Endogenous peroxidase was quenched using an aqueous solution of hydrogen peroxide (0.3%) at room temperature for 30 min. Sections were washed well in TBS and permeabilized using 0.1% Triton-X-100 for 10 min. Non-specific staining was blocked by incubation with non-immunized swine serum (10%) for 30 min at room temperature, prior to incubation with primary antibody overnight (17 ± 1 h) at 4°C. Antibodies were diluted to 0.75 µg/ml ($\alpha$ subunit), 2 µg/ml ($\beta$A subunit) and 1.2 µg/ml ($\beta$B subunit) in carrageenan diluent (0.7% carrageenan, 0.3% Triton-X-100 in TBS) containing 10% swine serum and 10% fetal calf serum. Negative controls were also included by the substitution of non-immunized rabbit IgG (X0936; Dako, Glostrup, Denmark) at a concentration matching the primary antibody and incubation with primary antibody overnight (17 ± 1 h) at 4°C. Antibodies were diluted to 0.75 µg/ml ($\alpha$ subunit), 2 µg/ml ($\beta$A subunit) and 1.2 µg/ml ($\beta$B subunit) in carrageenan diluent (0.7% carrageenan, 0.3% Triton-X-100 in TBS) containing 10% swine serum and 10% fetal calf serum. Negative controls were also included by the substitution of non-immunized rabbit IgG (X0936; Dako, Glostrup, Denmark) at a concentration matching the primary antibody. Antibody binding was thereafter detected by the sequential application of biotinylated swine anti-rabbit immunoglobulins (E0353; Dako) and avidin–biotin–peroxidase conjugate (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA). Positive localization was determined by application of the chromogen diaminobenzidine (DAB; Zymed, San Francisco, CA, USA), forming an insoluble brown precipitate. Tissue sections were counterstained with Harris’s haematoxylin, dehydrated in alcohol and mounted from histosol with DPX.

Positive immunostaining was scored semi-quantitatively by two independent observers, blind to the stage of the menstrual cycle, or time following levonorgestrel delivery. Scoring was analysed on the basis of the degree of staining and its localization within different components of the endometrium, and allocated a score between 0 (no staining), 1 (minimal staining), 2 (strong staining) or 3 (intense staining).

Co-localization studies
To determine the identity of $\beta$B subunit positive leukocytes, serial tissue sections (2 µm) were examined for the presence of specific leukocyte subpopulations. Immunostaining for $\beta$B subunit was conducted as described above. Leukocyte subpopulations were identified using antibodies directed against the specific subpopulations: macrophages (CD68, M0814; Dako), endometrial granular lymphocytes...
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In-situ hybridization

In situ hybridization was conducted using digoxigenin-labelled riboprobe to detect mRNA encoding inhibin α, βA and βB subunits. Riboprobes were synthesized from the same plasmid preparations as those used for synthesis of cDNA probes for Northern hybridization studies, using digoxigenin (DIG) RNA labelling mix (Boehringer Mannheim, Castle Hill, NSW, Australia) and were thereafter purified by centrifugation through Chromaspin columns (PT1300–1; Clontec, Palo Alto, CA, USA), quantified and stored at −80°C until further use.

Paraffin sections (5 µm) were dewaxed in histosol and rehydrated through ascending grades of ethanol. Tissue sections were thereafter permeabilized in a solution of 0.3% Triton-X-100, and digested with 12 µg/ml proteinase K for 30 min at 37°C. Following post-fixation with 4% paraformaldehyde for 10 min at 4°C, tissue sections were acetylated and incubated with prehybridization solution for 1 h at 42°C. Antisense and DIG-labelled probes were applied in hybridization buffer (containing 50% formamide, 1 × Denhardt’s solution, 10% dextran sulphate, 1 mg/ml rRNA and 0.1 mg/ml salmon sperm DNA) at the following concentrations (0.4 ng/ml α subunit, 0.2 ng/ml βA subunit, 0.3 ng/ml βB subunit), and incubated for 1 h at 42°C for inhibin α and βB subunits and 45°C for the βA subunit. After hybridization, tissue sections were washed in increasingly stringent solutions of SSC (2 × to 0.1 × SSC) containing 20% formamide at 42°C or 45°C in a shaking waterbath, and non-specific binding was abolished by treatment with 20 µg/ml RNase A for 30 min at 37°C. Specifically bound probe was subsequently visualized using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) diluted in blocking serum (10% normal sheep serum, 10% fetal calf serum, 0.1% Triton-X-100), with Nitro Blue tetrazolium/S-bromo-Δ-chloro-3-indolyl phosphate (K0598; Dako) as a chromogen, to produce a deep blue-purple stain. No counterstain was used and sections were mounted with glycerol gelatin (GG-1; Sigma Diagnostics, St Louis, MO, USA). Sense probes were applied at matching concentrations to serial tissue sections and treated identically to act as a negative control. Third trimester placenta was included as a positive control.

Results

Immunohistochemistry

Immunostaining was detected for all three inhibin subunits in the positive control tissues examined, localized specifically to the granulosa cells of rat ovary, and to varying degrees within the syncytiotrophoblast, amniochorion and decidua of third trimester placental tissue, in accordance with previously published data. A summary of the immunohistochemical data for each subunit in endometrium at different stages of the menstrual cycle endometrial group is shown in Table I.

α subunit

Inhibin α subunit was detected, with low and variable expression in non-pregnant endometrium, predominantly in the glandular and surface epithelium. With the onset of decidualization in the late secretory phase however, expression of α subunit was also detected in selected stromal cells exhibiting characteristics of decidual cells (Figure 1A–C; Figure 2), whilst glandular epithelial staining appeared to be reduced.

In decidua collected from early pregnancy, extensive α subunit immunostaining was detected throughout the decidualized stroma, frequently with an intense pericellular staining.
Table 1. Summary of immunohistochemical findings determining localization and intensity of immunostaining intensity for inhibin α, βA and βB subunits in human endometrium across the normal menstrual cycle and in early pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Proliferative</th>
<th>Late secretory</th>
<th>Early pregnancy</th>
</tr>
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<tbody>
<tr>
<td>Glandular epithelium</td>
<td>α +</td>
<td>α –</td>
<td>α –</td>
</tr>
<tr>
<td></td>
<td>βA +</td>
<td>βA + βA +</td>
<td>βA + + + +</td>
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<tr>
<td>Stroma</td>
<td>α – βA + α</td>
<td>βA +</td>
<td>βB + + + +</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>α – βA + βB</td>
<td>α – βA + βA +</td>
<td>βB + + + + + + +</td>
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= no staining; +/- = minimal staining; + = strong staining; ++ = intense staining.

Maximal numbers of leukocytes were detected in proliferative phase endometrium (Figure 1G), with declining numbers detected in the decidualized endometrium (Figure 2). Serial endometrial sections stained for βA and βB subunits revealed that βA-positive leukocytes were distributed throughout the functionalis (Figure 3A), whereas a different subpopulation of leukocytes, preferentially localized to the basalis, was positive for βB subunit (Figure 3B and E). Co-localization studies revealed that the cells expressing βB protein were a subset of macrophages (Figure 1M–N) and to a lesser extent, mast cells (Figure 1O–P), whereas all other leukocyte subpopulations examined (neutrophils, eosinophils, T lymphocytes and eGLs) did not appear to express the βB subunit.

βA and βB subunits

Both βA and βB subunits were detected in endometrium and showed a similar localization. Previous reports from our laboratory described βA localization in glandular and surface epithelium, with maximal immunostaining detected in late secretory endometria (Leung et al., 1998). Additional immunostaining for βA was detected in a subpopulation of uterine leukocytes. In the present study late secretory phase and first trimester decidua tissues were examined, and intense immunoreactivity was observed in the stromal compartment coincidental with decidual transformation (Figure 1D–F).

Immunoreactivity for the βB subunit was similarly identified in the epithelial component and further intense staining was apparent in a subset of stromal leukocytes. In late secretory phase endometrium displaying signs of pseudodecidualization, stromal cells clustered around spiral arterioles exhibited immunoreactivity for βB subunit (Figure 1G–I; Figure 2). This pattern was enhanced in first trimester decidua, with immunostaining present in all decidualized stromal cells while staining was maintained in glandular epithelium. Low numbers of stromal leukocytes expressing either βA or βB subunits were detected in first trimester decidua (Figure 2).

Leukocytes

Maximal numbers of βB positive leukocytes were detected in proliferative phase endometrium (Figure 1G), with declining numbers detected in the decidualized endometrium (Figure 2). Serial endometrial sections stained for βA and βB subunits revealed that βA-positive leukocytes were distributed throughout the functionalis (Figure 3A), whereas a different subpopulation of leukocytes, preferentially localized to the basalis, was positive for βB subunit (Figure 3B and E). Co-localization studies revealed that the cells expressing βB protein were a subset of macrophages (Figure 1M–N) and to a lesser extent, mast cells (Figure 1O–P), whereas all other leukocyte subpopulations examined (neutrophils, eosinophils, T lymphocytes and eGLs) did not appear to express the βB subunit.

Lng-IUS

The exposure to local high dose levonorgestrel induces rapid and extensive decidualization of the endometrium and abolishes all cyclical activity. All inhibin subunits (α, βA and βB) were markedly up-regulated in decidualized stromal cells following insertion of the Lng-IUS, corresponding to the extent of decidualization (Figure 1J–L). Both βA and βB subunits were maintained in glandular epithelium, in which the α subunit was distinctly absent (Figure 4).

Increased numbers of βA positive leukocytes were detected after insertion of the device, compared with the numbers detected before insertion and these remained elevated at all time points examined after insertion (Figure 4). Of interest were individual endometrial biopsies containing clusters of βA positive leukocytes, characteristic of neutrophil infiltration during acute inflammation (Figure 3C). Further, leukocytes producing βA subunits were preferentially localized to subepithelial regions and appeared to be adherent to the endometrium of blood vessels (Figure 3D). After an initial increase in βB positive leukocytes, reduced numbers were present post-insertion compared with proliferative phase biopsies before insertion of the Lng-IUS (Figure 4). Notably, no βB expressing leukocytes were identified in the extensively decidualized biopsies obtained 12 months after insertion of the Lng-IUS.

Norplant

Variable levels of immunostaining for all three subunits were observed in tissues collected from women exposed to systemic levonorgestrel via subdermal implants. Endometrial biopsies were subdivided on a histological basis into one of three categories: proliferative, progesterone-modified or shedding (Vincent et al., 1999), although it must be noted that the majority of biopsies displayed heterogeneous morphology and this is reflected by the varied immunostaining patterns obtained (Figure 5). Glandular α subunit immunostaining was maximal in biopsies classified as proliferating, compared to shedding or progesterone-modified endometria in which expression was much lower. As expected, biopsies displaying signs of decidualization exhibited elevated stromal immunostaining for α subunit. No differences in immunostaining intensity were observed for βA subunit in any of the Norplant endometrial groups examined, with prominent glandular and some decidual stromal immunostaining apparent. Prominent glandular immunostaining for βB subunit was observed in proliferative type endometria, whilst stromal immunostaining was detected only in the proliferative and progesterone modified biopsies. It is noteworthy that maximal βB immunoreactivity by uterine leukocytes was detected in those biopsies classified as proliferating compared with the decidualized or shedding biopsies, whilst relatively constant levels of βA-expressing leukocytes were detected in all tissues examined (Figure 5).

Northern hybridization

RNA transcripts of expected sizes were detected for inhibin α (1.6 kb), βA (6.0 kb) and βB (4.8 kb) subunits in the control tissues (bovine corpus luteum) (data not shown), human term placenta and HepG2 hepatoma cell line (Figure 6). The same
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Figure 1. Immunohistochemical localization of inhibin subunits in human endometrium. (A–C) Immunostaining for α subunit in (A) proliferative phase and (B) late secretory phase endometrium and (C) in first trimester decidua. Note alteration in immunostaining localization from predominently glandular in the proliferative phase to stromal in decidualized endometrium. (D–F) Immunostaining for βA subunit in endometrium, in (D and E) late secretory phase (arrowheads indicate presence of βA positive leukocytes) and (F) first trimester decidua. (G–I) Immunostaining for βB subunit in endometrium in (G) proliferative phase (arrowheads indicate presence of βB positive leukocytes), (H) late secretory phase endometrium and (I) first trimester decidua. (J–L) Immunohistochemistry for inhibin subunits post insertion of Lng-IUS. (J) α subunit expression 6 months post-insertion, note absence of immunostaining in atrophic glands, (K) βA immunostaining at 6 months post-insertion (arrow heads indicate presence of βA positive leukocytes) and (L) 12 months post-insertion. Intense immunostaining is observed throughout extensively decidualized endometrium. (M–P) Co-localization of βB subunit with leukocyte subtype markers. Arrows indicate cell staining positively for both markers. (M) βB and (N) CD68, immunostaining to recognize macrophages (O) βB subunit and (P) mast cell tryptase immunostaining. Scale bars = 100 µm. Scale bar on figure (L) relates to figures (A–L), scale bar on figure (P) relates to (M–P). ge = glandular epithelium, ds = decidualized stroma; le = leukocyte.

blot was also analysed for GAPDH mRNA expression levels to ensure all samples were equally loaded. Bands of comparable intensity were obtained for all endometrial tissues. However, GAPDH appeared to be expressed at a far greater level in the HepG2 cells, as previously observed for RNA derived from human liver (unpublished observations). For this reason, the 28S rRNA subunits as detected by gel electrophoresis were photographed prior to transfer to nylon membrane, and are included to demonstrate equal RNA loading.

The successful hybridization indicated that these probes were suitable for use for in-situ hybridization on human endometrial tissue sections. No signal was obtained for either the α or βB subunit in RNA extracted from non-pregnant or pregnant endometrium indicating that these were expressed at
a lower level than the sensitivity of this technique. However, a strong signal was obtained for βA subunit mRNA in endometrium collected at all stages of the menstrual cycle suggesting that this subunit is expressed at a higher level (proposed relative mRNA abundance: inhibin βA>βB and α subunits).

**In-situ hybridization**

In-situ hybridization analysis of selected endometrial samples revealed intense staining for all three inhibin subunits with a similar localization to that seen for the protein by immunohistochemistry (Figure 7). Specifically, α subunit mRNA was strongly localized to the glandular epithelium in proliferative (Figure 7A) and early secretory phase endometrium, with lower amounts of staining in foci scattered through the stroma. In decidualized tissues, both from the late secretory phase and from early pregnancy (Figure 7B–D), intense staining representing α subunit mRNA transcription was present throughout the decidualized stroma. Very low levels of α subunit mRNA were detectable in glandular and surface epithelium. Third trimester placenta included as a positive control exhibited staining in syncytiotrophoblast (Figure 7E). Sense controls showed no staining (Figure 7F).

Similarly, inhibin βA mRNA was detected predominantly in glandular epithelium in most non-pregnant endometrial tissues (Figure 7G), with strong stromal staining apparent following decidualization (Figure 7H–J). As seen for the βA protein, mRNA transcripts were also present in epithelial glands in the decidualized endometrium (Figure 7J). Extensively decidualized endometrium from women using Lng-IUS showed intense staining in epithelial and stromal elements (Figure 7K). Sense controls were completely negative (Figure 7L). An identical spatial pattern of staining was observed for inhibin βB as for βA subunit (Figure 7M–R). Proliferative and early secretory phase tissues exhibited glandular staining (Figure 7M–N), whilst widespread stromal staining was observed in first trimester decidua (Figure 7O) and after Lng-IUS insertion

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**Figure 2.** Histogram displaying immunohistochemical localization and relative staining intensity (mean ± SEM) for inhibin α and βB subunits in endometrial epithelial glands (white bars), stroma (hashed bars) and leukocytes (gray bars). Data is presented from all stages of the menstrual cycle: menstrual (M), proliferative (P), early secretory (ES), mid-secretory (MS), late secretory (LS), and early pregnancy: first trimester decidua (DE). Where no errors bars are shown, all values within the group were consistent.

**Figure 3.** Immunoreactivity for inhibin β subunits in endometrial leukocytes. (A) βA subunit positive leukocytes are detectable scattered throughout functionalis (insert (a) shows high magnification). (B) βB subunit immunostaining in leukocytes is limited primarily to basalis (insert (b) shows high magnification of functionalis, note that no βB positive leukocytes are visible). (C, D) Representative photomicrographs of βA-expressing leukocytes in endometrium following Lng-IUS insertion. (E) High magnification image of βB subunit positive leukocytes in functionalis-basalis junction. Scale bars = 100 μm. le = leukocyte, v = vessel, se = surface epithelium.
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(Figure 7P). Third trimester placenta exhibited staining in syncytiotrophoblast (Figure 7Q).

Discussion

All three inhibin/activin subunits investigated, α, βA and βB, were expressed by normal endometrial tissue. Immunoreactive protein was detected primarily in glandular epithelium in the earlier stages of the menstrual cycle, with elevated expression in the stroma corresponding to the onset of decidualization and continuing into early pregnancy. Interestingly, loss of staining for α subunit was consistently observed in glandular epithelium in decidualized tissues, while glandular staining for both β subunits was retained. The expression of all three subunits was also verified in extensively decidualized tissues from women using Lng-IUS and in decidualized regions of endometrial tissue from Norplant users. These patterns were identified both at the mRNA and protein level. Immunoreactivity for βA and βB subunits was also detected in specific subpopulations of endometrial leukocytes, predominantly subsets of uterine macrophages.

The up-regulation of inhibin subunits in decidualized stromal cells coincident with the progesterone-dominated post-ovulatory (secretory) phase, and also following administration of exogenous progestin (via Lng-IUS and Norplant), implies a putative regulation by progesterone. The restriction of stromal inhibin subunit expression only to regions of endometrium

![Figure 4. Immunostaining scores (mean ± SEM) for inhibin α, βA and βB subunits in endometrium following local delivery of progestin via Lng-IUS. Data is presented for tissue biopsied preinsertion of the Lng-IUS, either during the proliferative (P) or secretory (S) phase, and at 1, 3, 6 and 12 months post-insertion of the device. The upper panel represents the degree of decidualization at each time point (semi-quantitative analysis). Inhibin subunit staining is presented for both epithelial glands (white bars), stroma (hashed bars) and leukocytes (grey bars). Where no errors bars are shown, all values within the group were consistent.](image)

![Figure 5. Immunohistochemical localization and relative staining intensity (mean ± SEM) for inhibin α, βA and βB subunits in endometrium collected from women using Norplant. Data is presented for staining localized to epithelial glands (white bars), stroma (hashed bars) and leukocytes (grey bars). Endometrial biopsies were subdivided into three groups based on morphological appearance: proliferative, progesterone-modified and shedding. Where no errors bars are shown, all values within the group were consistent.](image)
by assessment of band intensity for the 28S rRNA subunit or secretory (S) phases of the normal menstrual cycle, and in mRNA in endometrial samples collected during the proliferative (P) probe against glyceraldehyde dehydrogenase (GAPDH) mRNA and by assessment of band intensity for the 28S rRNA subunit following gel electrophoresis.

Figure 6. Northern analysis of inhibin α, βA and βB subunit mRNA in endometrial samples collected during the proliferative (P) or secretory (S) phases of the normal menstrual cycle, and in first trimester decidua (De) and placenta (Pl). RNA extracted from the human hepatoma cell line (HepG2) is included as a positive control. RNA loading was assessed using a probe against glyceraldehyde dehydrogenase (GAPDH) mRNA and by assessment of band intensity for the 28S rRNA subunit following gel electrophoresis.

exhibiting signs of decidual transformation suggests an indirect regulation by the steroid, and we would propose that inhibins and activins are regulated indirectly by autocrine/paracrine factors produced during the decidualization process. This is reinforced by the expression patterns obtained following levonorgestrel delivery, with lower expression of inhibin subunits in endometrium from Norplant users compared with that observed following local Lng delivery. This relates directly to the reduced extent of decidualization following subdermal levonorgestrel delivery, compared with that observed following local Lng-IUS insertion (Silverberg et al, 1986; Vincent et al, 1999).

The lack of α subunits in glandular epithelium of the decidualized endometrium indicates preferential production of activin dimers in epithelial cells, while co-expression of α and β subunits by decidualized stroma confers potential for inhibin and activin dimer formation. This is in agreement with previous data (Petraglia et al, 1998), who demonstrated preferential production of dimeric activin A by cultured endometrial epithelial cells, whilst both epithelial and stromal cells produced inhibins A and B. Furthermore, activin A was detected at higher levels than either inhibin A or B (Petraglia et al, 1998), consistent with our Northern analysis data, which indicates the βA subunit is in far greater abundance than either the α or βB subunits. Since the majority of glandular derived products are secreted apically, it seems likely that the endometrium is a potential source of activin dimers present in the intrauterine compartment during early pregnancy (Riley et al, 1996; 1998). Roles for endometrially derived inhibins and activins may be envisaged in facilitating trophoblast differentiation (Caniggia et al, 1997) and steroidogenesis (Ni et al, 2000), and additionally in embryogenesis (He et al, 1999).

Decidualization represents a mesenchymal–epithelial transition, with widespread morphological and functional changes throughout the stroma and glandular epithelium (Loke and King, 1995). These changes are governed by ovarian steroid hormones, but important functions have been ascribed to locally acting mediators in effecting the transformation process. In this capacity, a number of actions of activins may be related to the changes occurring during decidualization. Activin A is a local regulator of cell differentiation and tissue remodelling, with well defined roles in wound healing (Hübner et al, 1999; Munz et al, 1999) and mesoderm induction during embryogenesis (Smith et al, 1990). Facilitatory roles in enhancing differentiation and proliferation of stromal (Oliver et al, 1999) and epithelial (Robinson and Hennighausen, 1997; Li et al, 1998) cell types have been described in other systems. Further, an integral role in decidualization has been proposed in rat decidua, where spatial and temporal expression of activin A is closely correlated with decidual transformation and apoptotic regression (Gu et al, 1995). At this time there is little relevant data regarding specific local functions of activin B derived from endometrial epithelial and stromal cells.

Paracrine actions of inhibins have been reported (for reviews, see Mather et al, 1992; Findlay, 1993), including modulation of ovarian and placental hormone secretion and local regulation of macrophage function (Hedger et al, 1989; Petraglia et al, 1991). Inhibins are generally considered to be functional antagonists of activins, indeed a local imbalance of α and β subunits, as demonstrated in the inhibin α subunit-deficient mouse, is related to gonadal tumorigenesis (Matzuk et al, 1992; Zheng et al, 1998). Since no functional signalling inhibin receptor has to date been identified (Woodruff, 1999), mechanisms of inhibin action are unclear. Inhibins may interact with activin receptors, thus antagonizing activin actions through competitive receptor binding (Xu et al, 1995). Further studies are required to determine the cellular location of activin receptors in the uterus.

Subpopulations of macrophages and neutrophils in the uterus express βA subunits (Leung et al, 1998; present study), with elevated numbers present in the late secretory phase. This coincides with a premenstrual infiltrate of leukocyte subpopulations into the endometrium, characteristic of the inflammatory type responses associated with endometrial breakdown during menstruation (Finn and Pope, 1984; Critchley et al, 1999). Levonorgestrel administration (either via Norplant or Lng-IUS) is associated with augmented inflammatory responses (Clark et al, 1996; Critchley et al, 1998; Vincent et al, 1999), and in this study we demonstrate the prominence of βA expressing leukocytes in these tissues. Activin A is associated with a number of inflammatory processes, including rheumatoid arthritis (Yu and Dolter, 1997), wound healing (Hübner et al, 1996) and inflammatory bowel disease, particularly localized to regions of epithelial destruction and inflammatory cell infiltrate (Hübner et al, 1997). Activin A mRNA expression is stimulated by proinflammatory mediators, including interleukin-1, tumour necrosis factor-α, interferon-γ, lipo polysaccharide (Yu et al, 1996; Shao et al, 1998) and is
induced during activation of peripheral monocytes in vitro (Erämaa et al., 1992).

Anti-inflammatory actions for activin A have been proposed, due to its ability to inhibit biological activities of interleukin-6, abolishing induction of the acute phase response (Sternberg et al., 1995; Shao et al., 1998). It seems likely that leukocyte-derived activin A is involved in modulating endometrial inflammatory responses associated with the perimenstrual period. Additionally, macrophage-derived activin A has been demonstrated to induce apoptosis of B-lineage hybridoma cells (Nishihara et al., 1995), signifying further potential paracrine actions of activin A released by accumulated macrophages within endometrial stroma during the premenstrual tissue breakdown.

To our knowledge, this is the first demonstration of βB subunit expression by leukocytes. Interestingly, βA and βB subunits are expressed by different subsets of uterine macrophages, showing distinct localization within the endometrium. Further, βB-positive macrophages are detectable primarily in the proliferative phase of the cycle and in tissues exhibiting a
proliferative type morphology following subdermal progesterin delivery. The abundance of macrophages and mast cells expressing βB subunits at the functional–basalis interface is coincidental with dramatic tissue reorganization and regeneration in this region following menses (Salamonsen and Lathbury, 2000). βB subunits are expressed by migrating keratinocytes and epithelium in actively repairing wounds, with expression extended during tissue remodelling when βA subunits, which are associated primarily with initial inflammatory events, cease to be expressed (Hübner et al., 1996). This implies a more important role for activin B in modulating tissue regeneration and remodelling. The distinct leukocyte expression patterns of βA and βB subunits within the endometrium indicate different functions for activin A and B within the tissue, potentially fulfilling functions associated with inflammation and tissue repair following menstruation.

This manuscript describes in detail the sites of production of the inhibin/subunits in the human endometrium and points to potential roles for the biologically active dimers in this tissue. A number of diverse functions may potentially be fulfilled by activins and inhibins in the uterus, particularly in facilitating tissue reorganization and remodelling, and modulating immune cell function during decidualization and menstruation.

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References

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