Presence of secretory leukocyte protease inhibitor in human endometrium and first trimester decidua suggests an antibacterial protective role

Anne E. King1,3, Hilary O.D. Critchley2 and Rodney W. Kelly1

1Medical Research Council Reproductive Biology Unit and 2Obstetrics and Gynaecology, University of Edinburgh, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9ET, UK
3To whom correspondence should be addressed

Secretory leukocyte protease inhibitor (SLPI) is a neutrophil elastase inhibitor which also has antibacterial and anti-inflammatory properties. It is found associated with mucosal membranes. Although SLPI has been reported in the cervix it has not thus far been reported in human endometrium. This study investigates the presence of SLPI in endometrium, first trimester decidua and trophoblast. Cultured first trimester decidua was found to produce 4.7 ± 2.0 ng/mg/24 h of SLPI. Endometrium and trophoblast were both found to secrete significantly lower amounts of SLPI (P < 0.01) although endometrial expression was menstrual cycle dependent with increased secretion in the secretory phase. Although relatively low concentrations of SLPI were released from the endometrium during culture, most of the SLPI remained associated with the tissue and could be recovered with mild acid extraction. This is in agreement with the high isoelectric point (pI) for SLPI, associated with high solubility at low pH. The main site of SLPI synthesis in endometrium and decidua was found to be the glandular epithelium. An antibiotic role for SLPI in the endometrium and decidua during implantation and pregnancy would be consistent with the expression profile and localization of SLPI.

Key words: defensins/endometrium/epithelial glands/natural antibiotics/SLPI

Introduction

Secretory leukocyte protease inhibitor (SLPI) is a 11.7 kDa cysteine-rich protein found associated with mucosal surfaces, e.g. lung and cervix (Franken et al., 1989). SLPI is produced by neutrophils (Bohm et al., 1992), macrophages (Jin et al., 1997) and epithelial cells (Abe et al., 1991). SLPI is thought to function mainly as a serine protease inhibitor (serpin) and has been characterized as a critical inhibitor of neutrophil elastase in the lung (Thompson and Ohlsson, 1986). There is evidence to suggest that SLPI has a protective function at mucosal membranes. SLPI has been shown to kill both Escherichia coli and Staphylococcus aureus, thus showing bactericidal activity against gram-negative and gram-positive bacteria (Hiemstra et al., 1996). Antiviral (McNeely et al., 1995) and antifungal (Tomee et al., 1997) effects have also been observed. Secondly, evidence suggests that SLPI acts to inhibit various proinflammatory systems. For example, in mouse macrophages SLPI inhibits the actions of lipopoly-saccharide (LPS) (Jin et al., 1997). Also in human monocytes, SLPI decreases the production of matrix metalloproteinases (responsible for extracellular matrix degradation) via suppression of a cyclo-oxygenase-2/prostaglandin E2/cAMP pathway (Zhang et al., 1997). Receptors for SLPI have been identified in human monocytes (McNeely et al., 1997). Finally, SLPI inhibits activation of the NFκB signal transduction pathway (Jin et al., 1997) which is a major inducer of proinflammatory genes. SLPI is thus a pleiotropic molecule protecting against inflammatory insult and infection in a variety of ways.

In a reproductive context, SLPI has been detected in human cervical mucosa (Casslen et al., 1981), term decidua (Denison et al., 1999) and seminal plasma (Franken et al., 1989). It has also been localized to the glandular and luminal epithelial cells of maternal endometrium of pig (Reed et al., 1996) and the mRNA has been detected in horse and cow endometrium during pregnancy (Badinga et al., 1994). It has been suggested that SLPI acts to maintain the uterine–placental border in these species and that expression is related to epitheliocorial placentaion. SLPI has not been detected in the endometrium of mammals with haemochorial placentaion (e.g. rat) (Badinga et al., 1994) and previous studies have failed to detect the protein in non-pregnant human endometrium (Casslen et al., 1981; Franken et al., 1989), although SLPI has been found to be present in uterine fluid (Casslen et al., 1981). Successful human pregnancy demands that inflammatory responses in the uterus are suppressed and, as an antibacterial and anti-inflammatory molecule, it seems probable that SLPI may contribute to this. This study investigates the expression of SLPI by human endometrium, decidua and trophoblast.

Materials and methods

Tissue collection

Endometrial biopsies were collected from women undergoing gynaecological procedures for benign conditions. No attempt was made to distinguish between subjects with normal menstrual loss and undergoing a laparoscopic procedure for sterilization and subjects describing heavy menstrual loss and undergoing investigation or treatment (hysterectomy) for this subjective complaint. All women reported regular menstrual cycles (25–35 days) and had not received...
any form of hormonal treatment in the 3 months preceding biopsy. Biopsies were dated from the patient’s last menstrual period (LMP); histological dating according to published criteria (Noyes et al., 1950) was consistent with the date of LMP. Furthermore, circulating sex steroid concentrations were in keeping with the time in the cycle during which the biopsy was collected. Serum was separated from venous blood samples collected at the time of biopsy. Oestradiol and progesterone concentrations were measured by radioimmunoassay. The inter-assay coefficients of variation for the oestradiol and progesterone assays were 11.0 and 9.0% respectively, while the intra-assay coefficients of variation were 8.0 and 7.0% respectively. Oestradiol concentrations were in the range 0–4.45 pmol/l in the proliferative phase; all progesterone concentrations were <6 nmol/l. In the secretory phase, oestradiol concentrations were in the range 65–740 pmol/l; progesterone concentrations were >6 nmol/l (with the exception of two premenstrual samples).

In addition, endometrium was collected from women using a levonorgestrel intrauterine system (LNG–IUS; n = 4) for contraception and heavy menses. The LNG–IUS causes marked decidualization of the endometrium (Critchley et al., 1998).

First trimester decidua were collected by curettage of the uterine wall away from the site of implantation prior to suction termination of pregnancy. Trophoblastic villi were also collected during the procedure. Decidual parietalis (without trophoblast) was subsequently confirmed by examination of haematoxylin and eosin-stained sections.

Tissue samples were collected in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Poole, Dorset, UK); in addition, endometrial biopsies were fixed in 1% neutral-buffered formalin (NBF) overnight at 4 °C, stored in 70% ethanol, and then embedded in wax. Written informed consent was received from all patients prior to biopsy collection and ethical approval was received from Lothian Research Ethics Committee.

### Tissue culture

All tissue was cultured for 24 h on sterilized polypropylene capillary matting in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (Mycoplex, PAA Laboratories, Teddington, UK), penicillin (50 µg/ml; Sigma), streptomycin (50 µg/ml; Sigma) and gentamycin (5 µg/ml; Sigma). Endometrium and decidua were cultured in the presence of oestradiol (10 µmol/l). The culture medium was removed after 24 h for subsequent inclusion in SLPI assays. Tissue was weighed after incubation and all assay measurements were corrected for weight.

### Acid extraction of tissue

SLPI is an acid-stable molecule with a high isoelectric (pI) point and, therefore, more soluble in weak acid solution. After 24 h culture, tissue was acid extracted in the presence of 200 µl phosphate-buffered saline (PBS) and 10 µl HCl (1 N) for 10 min. Neutralization was achieved by addition of 10 µl NaOH (1 N) and 50 µl Tris buffer (1 mol/l; pH 7.2). Supernatant was collected for subsequent inclusion in SLPI assays.

### SLPI assay

SLPI was measured by an enzyme-linked immunosorbent assay (ELISA). Assay plates (96-well; Nunc Maxi-Sorp, Gibco, Paisley, UK) were coated with 0.025 µg/ml recombinant SLPI (R&D Systems, Oxford, UK) in PBS and 1% 400 mmol/l carbonate buffer. 100 µl were added to each well. Plates were left for 60 min at room temperature. Blocking was carried out with 400 µl well/blocking/protecting solution [polyvinylpyrrolidone 2%, bovine serum albumin (BSA) 5 mg/ml, preservatives, EDTA 5 mmol/l, Tris 50 mmol/l] for 30 min. Plates were washed with wash buffer (150 mmol/l NaCl, 100 mmol/l Tris, 0.05% Tween-20; pH 7–7.5). 150 µl of standard/sample and 50 µl of anti-SLPI (R&D Systems); 2 µg/ml; diluted in ELISA buffer (150 mmol/l NaCl, 100 mmol/l Tris, 50 mmol/l Phenol Red solution, 2 mmol/l EDTA, 1 mmol/l 2-methylisothiazalone) (Boehringer Mannheim, Lewes, UK), 1 mmol/l bromonitrodioxide (Boehringer Mannheim), 2 mg/ml BSA, 0.05% Tween-20; (pH 7.2) were added to each well. A non-specific binding well (200 µl buffer only) and two B0 wells (50 µl anti-SLPI) were included on each plate. Standards were added in triplicate and their concentration range was from 50–0.098 ng/ml. Incubation was on a plate shaker at room temperature for 2 h; and the plates were then washed. 100 µl/well of peroxidase-labelled anti-sheep/goat immunoglobulin G (IgG) Fab fragments raised in donkey (Boehringer Mannheim; 1:1000 dilution of stock in ELISA buffer) were added. Incubation was on a shaker as before and the plates were washed again. 200 µl substrate (0.3 g/l urea–hydrogen peroxide, 0.1 g/l tetramethyl benzidine in 100 mmol/l sodium acetate; pH 6) were added to each well. After 2–10 min, wells were quenched with 50 µl/well 2 N sulphuric acid. Plates were read in a plate reader at 450 nm.

### Immunohistochemistry

Tissue sections were dewaxed in histoclear (National Diagnostics, Atlanta, Georgia, USA) and rehydrated in descending grades of alcohol. Non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (BDH Laboratory Supplies, Poole, UK) in distilled water for 10 min at room temperature. All tissue sections were subjected to a non-immune block with diluted normal horse serum (Vectastain 4002; Vector Laboratories, Peterborough, UK) for 20 min in a humidified chamber at room temperature. Tissue sections were then incubated overnight at 4 °C with 50 µl of goat anti-SLPI antibody (1:400 in horse serum; R&D Systems). In negative control sections the primary antibody was substituted with goat immunoglobulin (R&D Systems). Sections were then incubated with biotinylated horse-anti goat IgG (Vector Laboratories) followed by an avidin–biotin peroxidase detection system (both for 60 min at room temperature; Elite ABC 6101; Vector Laboratories). The peroxidase substrate diaminobenzidine (DAB; Vector Laboratories) was used to identify positive staining. Sections were then counterstained with Harris’s haematoxylin (R&D Systems). The mixture was divided into aliquots in individual tubes (8 µl/tube) and template RNA was added (2 µl RNA). The mix was divided into aliquots in individual tubes (8 µl/tube) and template RNA was added (2 µl RNA). Mineral oil was added and samples were incubated for 60 min at 25 °C, 45 min at 48 °C and then at 95 °C for 5 min.

### SLPI reverse transcription–polymerase chain reaction (RT–PCR)

Tissue samples were immersed in Ultraspec (Ultraspec RNA Isolation System, Biogenesis Ltd, Poole, UK), homogenized and RNA was extracted as detailed in the manufacturer’s protocol.

Decidual and endometrial RNA samples were reverse transcribed using random primers with MgCl2 (5.5 mmol/l), dNTPs, random hexamers (2.5 µmol/l), RNase inhibitor (0.4 IU/µl) and multiscribe reverse transcriptase (1.25 IU/µl; all from PE Biosystems, Warrington, UK). The mix was divided into aliquots in individual tubes (8 µl/tube) and template RNA was added (2 µl/tube of 100 ng/µl RNA). Mineral oil was added and samples were incubated for 60 min at 25 °C, 45 min at 48 °C and then at 95 °C for 5 min.

A reaction mix was made containing Taqman buffer, MgCl2 (5.5 mmol/l), dATP (200 µmol/l), dGTP (200 µmol/l), dUTP (400 µmol/l), ribosomal 18S forward and reverse primers and probe (all at 50 nmol/l), SLPI forward and reverse primers (both 300 nmol/l), SLPI probe (200 nmol/l), AmpErase UNG (0.01 IU/µl) and AmpliTag Gold DNA polymerase (0.025 IU/µl; all from PE Biosystems). The mixture was divided into aliquots in...
Endometrial SLPI

Figure 1. (a) Secretory leukocyte protease inhibitor (SLPI) production by control endometrium (throughout cycle), endometrium from levonorgestrel–intrauterine system users (LNG–IUS), decidua and trophoblast ($^{a,b}p < 0.01$). Numbers in the groups are shown immediately above the error bars. (b) SLPI production by control endometrium from the proliferative, early/mid-secretory (ES/MS) and late secretory (LS) phases of the menstrual cycle ($^{a,b}p < 0.0001$).

Figure 2. Secretory leukocyte protease inhibitor (SLPI) release during acid extraction of control endometrium, decidua and trophoblast ($^{a,b}p < 0.05$).

Results

SLPI secretion by endometrium, decidua and trophoblast

Decidua ($n = 17$) produces 4.7 ± 2.0 ng/mg of SLPI. This is significantly higher ($P < 0.01$) than secretion by either endometrium or trophoblast which produce 0.4 ± 0.1 ng/mg and 0.1 ± 0.04 ng/mg respectively. Endometrium from LNG–IUS users produces 1.4 ± 1.0 ng/mg. This is similar to production by control endometrium (Figure 1a).

SLPI secretion by endometrium increases in the late secretory phase of the menstrual cycle. Secretion is 1.5 ± 0.2ng/mg at this time, compared with 0.2 ± 0.02ng/mg in the proliferative phase and 0.3 ± 0.1ng/mg in the early/mid-secretory phase (Figure 1b; late secretory compared with proliferative and early/mid-secretory, $P < 0.0001$).

SLPI release from endometrium, decidua and trophoblast during acid extraction

Endometrium releases 1185 ± 298% (of SLPI in control culture medium) during acid extraction whereas decidua releases only 150 ± 43%. Release from trophoblast (210 ± 72%) is also lower than from endometrium. (Figure 2; $P < 0.05$). This suggests that the majority of SLPI produced by endometrium is not secreted into the culture medium. The result from one decidual sample was discounted from this analysis as the value was 11805% (i.e. 94 SD from the mean of the other values).

Localization of SLPI in endometrium and decidua

Immunohistochemistry showed positive SLPI immunoreactivity in the glandular epithelium and secretions in endo-
metrial biopsies from the mid to late secretory phase. Immunostaining was also present in the luminal epithelium of some biopsies. The immunoreactivity was mainly localized to superficial areas of endometrium. No stromal staining occurred (Figure 3b). Very little SLPI was detected in proliferative and early secretory phase biopsies (Figure 3a).

SLPI immunostaining was also present in the epithelial cells of the glands and their secretions in decidua biopsies. Immunoreactivity was also found in the stromal cells of some decidua (Figure 3c).

**SLPI RT–PCR**

The accuracy of the quantitative PCR was tested by serial dilution (to 64× dilution) of a pool of SLPI cDNA. The slope of the line plotting the cycle number at which the curve crossed a threshold (Ct) against dilution had a gradient <0.1ΔCt units/64× dilution. Intra-assay precision of the PCR was calculated as 9.7% (r.s.d.). All data were obtained from a single PCR run and related to a standard SLPI cDNA preparation using the formula 2−ΔΔCt which relates the ratio of 18S and specific amplicon in the sample cDNA with that of the standard preparation.

SLPI mRNA was detected in all samples tested. Decidua contained 0.6 ± 0.3 of SLPI mRNA. This was greater than both proliferative and secretory endometrium which had 0.2 ± 0.1 and 0.5 ± 0.2 respectively (Figure 4a; not significant). Figure 4b shows that only one PCR product (representing SLPI) is amplified by the SLPI primers.

**Discussion**

To our knowledge this is the first report describing detection of SLPI in human endometrium and first trimester decidua. Previously, immunohistochemistry has failed to detect SLPI in human endometrium in either the proliferative or secretory phase of the menstrual cycle. However, SLPI was found to be present in uterine fluid and this was thought to be due to diffusion from the cervix (Casslen et al., 1981). It now seems likely that the source of this SLPI was endometrial glandular epithelial cells. This is consistent with the production of SLPI by epithelial cells at other mucosal surfaces such as lung. SLPI has previously been detected in term decidua where it was found to be localized to the large decidualized stromal cells (Denison et al., 1999). In first trimester decidua, SLPI was expressed mainly in the glandular compartment of the tissue although some stromal cells did contain SLPI. This suggests that as pregnancy progresses the site of SLPI production changes from the glandular epithelium to the stromal cells. A similar change of location has been reported in the case of the endothelin B (ETB) receptor. This receptor is found...
in the glandular epithelium in secretory endometrium but at the time of menstruation and in decidualized tissue the receptor is also present in stromal cells (Kohnen et al., 1998). It may be that as glands become atrophied as decidualization progresses it is necessary for the stromal cells to produce glandular products in order to maintain decidual function. Trophoblast was found to secrete very little SLPI and is unlikely to be a major source during pregnancy.

In non-pregnant endometrium, SLPI secretion shows cycle dependence with an increase in the mid to late secretory phase. This suggests progesterone regulation of SLPI although it is likely that this effect is indirect rather than a direct action of progesterone on SLPI gene expression. For example, changes to glandular morphology (e.g. increased surface area) occur and there are variations in inflammatory mediator expression under the influence of progesterone. Such effects may lead to secondary changes in endometrium, e.g. increased SLPI production. SLPI expression increases particularly in the late secretory phase around the time of decidualization and implantation and then increases further in decidua. The amounts of mRNA present are consistent with the protein expression pattern found. Leukocytes infiltrate the endometrium prior to menstruation and may be responsible for the rise in SLPI in the late secretory phase. However, this would be inconsistent with the increased presence of SLPI in decidua. Additionally, immunostaining suggested that the main source of SLPI in endometrium is the glands. Decidualization may be a contributory factor in SLPI production. LNG–IUS users have endometrium which is decidualized due to the very high local concentrations of progestogen (Pekonen et al., 1992). Levonorgestrol-treated endometrium was found to produce higher concentrations of SLPI than proliferative endometrium but similar to those of late secretory endometrium.

Acid extraction of tissue was performed to ensure that the SLPI values measured in culture medium were a true representation of SLPI production by the tissue. Acid extraction of tissue resulted in release of SLPI from endometrium at concentrations much greater than those found during culture. Decidua and trophoblast released similar amounts of SLPI during culture and acid extraction. This suggests that SLPI production by endometrium far exceeds secretion although this may reflect in-vitro culture conditions. SLPI has been found to bind to endometrial extracellular matrix (Reed et al., 1997) and it may be that acid extraction is causing release of bound protein.

As a potent neutrophil elastase inhibitor, SLPI may have a role in the prevention of tissue degradation in the endometrium. Neutrophils infiltrate the tissue during menstruation (Poropatich et al., 1987) and may contribute to tissue breakdown. SLPI could act to limit this although it seems unlikely due to: (i) the superficial localization of the mediator; and (ii) the increased release of SLPI in pregnant decidua.

It has been suggested that in mammals which have epitheliochorial placentation, SLPI acts in maternal endometrium to maintain the integrity of the uterine–placental border by preventing inappropriate trophoblast invasion (Badinga et al., 1994). In humans, trophoblast cells invade throughout the decidua and into the myometrium, so SLPI is unlikely to regulate this invasion because of its localization primarily in the superficial endometrium.

SLPI has been found to have antibacterial, antiviral and antifungal effects and it has previously been suggested that its presence in the cervix and the male genital tract (with high concentrations in semen) protects these mucosal membranes from insult (Ohlsson et al., 1995). Endometrial production of SLPI could offer similar protection against infection of the uterus around the time of implantation and during pregnancy. Ascending infection is a major cause of premature labour, so natural antibiotics are likely to be important in controlling this. The location and expression profile of SLPI is consistent with an antibacterial role during pregnancy.

The defensins are a group of proteins found to have antibacterial, antifungal and antiviral actions. Defensin 5 and β-defensin 1 have been identified in epithelial cells of the endometrium and endocervix (Quayle et al., 1998; Valore et al., 1998). In addition, defensin 5 expression was found to be maximal in endometrium during the secretory phase of the

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**Figure 4.** (a) Amounts of secretory leukocyte protease inhibitor (SLPI) mRNA in decidua and proliferative and secretory endometrium. (b) Agarose gel of polymerase chain reaction (PCR) products showing only one amplification product. Lanes 1–3 show no template controls. Lanes 4–6 show replicate samples from one patient containing SLPI. The product corresponds to the product length expected for SLPI (~110 bp). Lane 7 contains PCR markers.

**Figure 5.** Comparison of secretory leukocyte protease inhibitor (SLPI) (upper sequence) and human β-defensin 1 (lower sequence) amino acid sequence.

<table>
<thead>
<tr>
<th>RRRPRGKCPVTQYQQCLMLNPKDFCQSDQCKRDL</th>
<th>RCCMGMCGRSGCSVPGKA</th>
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<tr>
<td>RSRHYCVSGDQQCLYSACPFTKQHTCYGRARKC</td>
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cycle (Quayle et al., 1998). One area of the SLPI molecule has 37% homology with the defensins and five or six cysteine residues involved in disulfide bonding are conserved between the proteins (Figure 5). Interestingly this area of homology does not coincide with the N-terminus of the SLPI molecule which is thought to be involved in antibacterial activity (Hiemstra et al., 1996). The similarities in protein sequence and expression of SLPI and the defensins gives further support to an antibacterial role for SLPI in endometrium.

Some bacteria secrete proteins which degrade SLPI implying that without this breakdown SLPI would pose a risk to the pathogen, Trichomonas vaginalis, a major pathogen of the lower female genital tract, releases cysteine proteases which degrade recombinant SLPI under assay conditions (Draper et al., 1998). It has been reported that there may be an association between irregular menstrual bleeding and the presence of bacteria in the uterus (Kristiansen et al., 1987; Moller et al., 1995). Our study does not attempt to distinguish between patients with normal and heavy menstrual loss. Indeed, all patients included in the present study reported regular menstrual cycles. Further study is required to determine concentrations of SLPI in endometrium during uterine infection or in the context of aberrant menstrual bleeding patterns.

In addition to the antibacterial effects, SLPI also inhibits the NFkB signal transduction pathway (Jin et al., 1997). This is a major pathway involved in inflammatory response. SLPI may have anti-inflammatory actions in decidua via inhibition of NFkB. This would prevent inappropriate inflammatory mediator expression during pregnancy.

In summary, SLPI has been detected for the first time in human non-pregnant endometrium and first trimester decidua. The most likely role of SLPI in this location is as a natural antibiotic and anti-inflammatory molecule. Infection ascending through the cervix could pose a threat to the implanting and developing conceptus. Indeed, the presence of high concentrations of SLPI in decidua might be some compensation for the immunosuppressive factors present in decidua of pregnancy.

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References


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