Altered secretory leukocyte protease inhibitor expression in the uterine decidua of tubal compared with intrauterine pregnancy

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BACKGROUND: The role of the innate immune system in tubal implantation remains undefined. This study compared expression of two key mediators of innate immunity, secretory leukocyte protease inhibitor (SLPI) and elafin, in the uterine decidua of women with intrauterine and tubal pregnancies. METHODS: Uterine decidua was collected from women (18–45 years) undergoing surgical termination of pregnancy (n = 7), surgical management of spontaneous abortion (n = 6) and tubal pregnancy (n = 10). Using quantitative RT–PCR and immunohistochemistry, mRNA and protein expression patterns of SLPI and elafin were compared. RESULTS: Relative SLPI mRNA expression was significantly higher in decidua of women with tubal pregnancy (12.37 ± 2.66) compared with spontaneous abortion (5.09 ± 2.22, P < 0.0185). There was no difference demonstrated in elafin mRNA expression. SLPI and elafin protein expression were demonstrated in the decidual leukocyte populations and epithelium. There was no obvious qualitative difference in levels of SLPI and elafin protein expression or their distribution in the uterine decidua of women with termination of pregnancy, spontaneous abortion or tubal pregnancy. CONCLUSIONS: Herein we demonstrate novel differences in gene expression of uterine decidua of tubal pregnancy compared with spontaneous abortion thereby contributing further to current knowledge of mechanisms involved in extraterine implantation. The altered expression of SLPI may be a consequence of, or predispose to, tubal pregnancy.

Keywords: secretory leukocyte protease inhibitor; elafin; uterine decidua; tubal pregnancy

Introduction

Tubal pregnancy is an important cause of maternal morbidity and occasional mortality. One in every 80 pregnancies is ectopic, with over 98% occurring in the fallopian tube (Farquhar, 2005). Understanding the innate immune mechanisms surrounding tubal implantation has obvious implications in the development of preventative public health strategies.

The immune system of the female reproductive tract has to maintain an infection-free environment while accommodating important mechanisms such as menstruation, fertilization, implantation, pregnancy and parturition (Finn, 1994; Wira et al., 2005). The adaptive immune system is principally involved in the removal of pathogens via the activation of antigen-presenting cells and the stimulation of T-cells. The innate immune system serves as the first line of protection, charged with both minimizing the spread of an infection and restricting the damage from a resulting inflammatory reaction (Medzhitov and Janeway, 2000; Alberts et al., 2002; Hoebe et al., 2004).

The identification of Toll-like receptors (TLRs) in the fallopian tube, uterus, cervix and vagina and the presence of epithelial cells, macrophages, dendritic cells, natural killer (NK) cells and neutrophils throughout the reproductive tract (along with their responsiveness to selected pathogen-associated molecular patterns) indicate that the female reproductive tract has evolved to meet the challenge of supporting an immunologically distinct fetal–placental unit within the uterus while maintaining its response to sexually transmitted infections (Medzhitov, 2001; Aflatoonian and Fazeli, 2008). However, the mechanisms controlling the level of
inflammation and recruitment of immune mediators in response to implantation at an ectopic site, such as the fallopian tube, are unclear.

During inflammation, a number of mediators and effectors of immunity are recruited to aid resolution and an over-exuberant, or prolonged, response can result in tissue damage. Among the mediators responsible for such tissue damage are proteases, which function as part of the innate immune response (Dallegri and Ottonello, 1997). The host response also includes the production of a number of important anti-proteases, such as the anti-microbials secretory leukocyte protease inhibitor (SLPI) and elafin, in order to counteract the actions of proteases and therefore prevent resultant damage to host tissues (Sallenave et al., 1994; Schalkwijk et al., 1999; Sallenave, 2000). SLPI inhibits a number of proteases, including neutrophil elastase, trypsin and cathepsin G, whereas elafin appears to be restricted to regulating neutrophil elastase and proteinase 3 (Thompson and Ohlsson, 1986; Sallenave and Ryle, 1991; Wiedow et al., 1991).

SLPI and elafin are expressed throughout the female genital tract (King et al., 2000, 2003b). The human endometrial epithelium expresses SLPI mRNA and protein during the secretory phase of the cycle and the neutrophils in the endometrium are a particularly rich source of elafin mRNA and protein during menstruation (King et al., 2003a). SLPI and elafin protein expression have also been demonstrated in the cervix and decidualized endometrium (Helimg et al., 1995; Pfundt et al., 1996; King et al., 2000, 2003a). The expression of SLPI and elafin during the menstrual cycle may indicate a role in tissue remodelling and ‘wound’ healing (van Bergen et al., 1996; Wingens et al., 1998; Ashcroft et al., 2000; Zhu et al., 2002; Angelov et al., 2004). In common with the rest of the female reproductive tract, the fallopian tube morphology undergoes cyclical changes under the influence of estrogen and progesterone (Sulz et al., 1998), and SLPI mRNA expression has been shown to have a temporal expression pattern in the fallopian tube similar to the endometrium (Horne et al., unpublished results).

However, changes in SLPI and elafin expression that may occur in the context of tubal implantation have not been demonstrated. Direct comparison of tubal with intrauterine sites is difficult due to the anatomical differences of the two implantation sites. However, similar to the endometrial response to an intrauterine pregnancy, with a tubal gestation, there is a decidual reaction in the uterine cavity but not usually in the fallopian tube (Stock, 1991). Decidualization of the endometrial epithelium and stroma is a key immunological element for successful implantation (Christian et al., 2002). The uterine decidua contains innate immune leukocytes, such as CD56+ NK (uNK) cells, macrophages and mast cells (Bulmer et al., 1988; King and Loke, 1991; Hunt, 1994; Marx et al., 1999). We therefore compared SLPI and elafin expression in the uterine decidua of women with ongoing intrauterine (women undergoing surgical termination of pregnancy, STOP), failed intrauterine (women with spontaneous abortion) and tubal gestations. Quantitative RT–PCR and immunohistochemistry were used to demonstrate mRNA and protein expression, respectively.

**Materials and Methods**

**Tissue collection**

Ethical approval for this study was obtained from Lothian Research Ethics Committee (04/S1103/20). Written and informed consent was obtained from all patients before sample collection. Uterine decidua and serum samples were obtained from women (age 18–45 years) undergoing STOP (n = 7, Group 1), surgical management of embryonic missed spontaneous abortion (n = 6, Group 2) and surgical management of tubal pregnancy (n = 10, Group 3). None of the women undergoing surgical management of tubal ectopic pregnancy presented acutely with haemodynamic shock, and all required serial serum beta-HCG and ultrasound monitoring prior to diagnosis. The decidua and trophoblast were obtained by suction curettage from Groups 1 and 2. The decidua was obtained by Pipelle endometrial biopsy from Group 3. The decidua was isolated from the trophoblast (Ambion, TX, USA) at 4°C overnight or flash frozen at −70°C and part (ii) fixed in 10% neutral buffered formalin over-night at 4°C, stored in 70% ethanol and wax embedded for immuno-histochemistry. Serum samples were stored for analysis of progesterone levels (protocol adapted from Corker and Davidson, 1978).

**Separation of decidua from trophoblast**

Decidual parietalis (decidua without trophoblast) was confirmed by haematoxylin and eosin staining and immunolocalization of cytokeratin in all biopsies prior to PCR/RT–PCR. A standard immunohistochemical protocol was used. Tissue sections were incubated with mouse anti-cytokeratin primary antibody (1:60, Dako, Ely, UK) for 1 h at 37°C followed by incubation with biotinylated horse anti-mouse (1:2000) and ABC (both Vector, Peterborough, UK) for 30 min at room temperature.

**Chlamydia trachomatis detection by PCR**

DNA was extracted from the decidual biopsies as detailed in the manufacturers’ protocol (Qiagen, West Sussex, UK). The PCR protocol used in-house plasmid-based methodology (kindly developed and designed by the West of Scotland Specialist Virology Centre).

**Quantitative real-time PCR**

Total RNA was extracted from the decidual biopsies as detailed in the manufacturers’ protocol (Qiagen, West Sussex, UK). The concentration and quality of the extracted RNA was assessed using an agilent bioanalyzer. All samples were standardized for quality control and assigned an RNA integrity number (RIN). RNA samples were considered to be of good quality when a mean RIN value of 7.5 was obtained (Schroeder et al., 2006). SLPI and elafin levels were determined by quantitative real-time PCR. This technique relates the amount of SLPI and elafin mRNA present to levels of ribosomal 18S, controlling for the amount of RNA present. Details of the RT and PCR methods have been fully detailed elsewhere (King et al., 2000). The elafin and SLPI primers and probe were used were previously designed in our laboratory using PRIMER express software (PE Biosystems, Foster City, USA) (King et al., 2000, 2003b). Elafin primers and probe were as follows: forward primer 5'-TGCCGTTGGGCTTGACATG-3', reverse primer 5'-CATGATCTTCTTAGGAGC-3' and probe 5'-ATCCGGTGCCATGTTTGATGC-3'. SLPI primers and probe were as follows: forward primer 5'-GCATCAACTTGCCATCAGTC-3' and probe 5'-TGACACCCCAAGGCCCAAGAGGAGG-3'. Within-assay variation of PCR measurements were calculated from six replicates. The variability of the RT step
was determined by reverse transcribing one RNA sample on eight separate occasions. The eight complementary DNA samples were then included within one PCR run, and variability (relative to SD) was calculated. The possibility of genomic DNA contamination was excluded by DNAse treatment and by measurement of beta-actin levels in RNA samples (which were not reverse transcribed). The relative mean of the samples from each clinical group was logged and analysed by analysis of variance (ANOVA) followed by Tukey post hoc analysis.

**Elafin immunohistochemistry**

Tissue sections were dewaxed in xylene and then rehydrated in descending grades of alcohol. Non-specific endogenous peroxidase activity was blocked with 2% hydrogen peroxide (Sigma Aldrich, Poole, UK) in distilled water for 10 min at room temperature. Diluted normal goat serum [20% vol/vol in phosphate-buffered saline (PBS)] (Diagnostics Scotland, Edinburgh, UK) was applied to all tissue sections for 20 min at room temperature. Avidin–biotin and protein blocks were further applied to the tissue sections for 20 min each at room temperature with alternate 5 min PBS washes. Sections were then incubated overnight at 4°C with rabbit anti-elafin polyclonal antibody (Sallenave et al., 1994) which was diluted at 1:700 in antibody diluent (Dako, Ely, UK). In negative control sections, the primary antibody was substituted with an approximately equivalent immunoglobulin (Ig) concentration of rabbit IgG (RLIgG; Vector Laboratories, Peterborough, UK). Sections were incubated with biotinylated goat anti-rabbit Ig (Vector) and were then subjected to an avidin–biotin peroxidase detection system (Vector; both were incubated for 30 min at room temperature). The peroxidase substrate, diaminobenzidine, was used to identify positive immunostaining. Sections were counterstained with Harris’ haematoxylin (Pioneer Research Chemicals, Colchester, UK), dehydrated in ascending grades of alcohol and mounted from xylene in Pertex (Cell-path, Hemel Hempstead, UK).

**SLPI immunohistochemistry**

The method used was identical to the elafin immunohistochemistry protocol, with the following exceptions. The non-immune block was performed with diluted horse serum (Vector) for 20 min at room temperature. The primary antibody was mouse anti-SLPI (Hycult Biotechnology, Uden, The Netherlands) diluted at 1:20 in antibody diluent (Vector). Negative controls were incubated with an equimolar concentration of rabbit IgG (RLIgG; Vector Laboratories, Peterborough, UK). Sections were incubated with biotinylated horse anti-mouse Ig (Vector) and were then subjected to an avidin–biotin peroxidase detection system (Vector; both were incubated for 20 min at room temperature). The peroxidase substrate, diaminobenzidine, was used to identify positive immunostaining. Sections were counterstained with Harris’ haematoxylin (Pioneer Research Chemicals, Colchester, UK), dehydrated in ascending grades of alcohol and mounted from xylene in Pertex (Cell-path, Hemel Hempstead, UK).

**Results**

**Serum progesterone levels**

Serum progesterone levels were analysed for each patient. There was no statistically significant difference between the groups using Kruskall–Wallis followed by Dunn’s post hoc analysis (Table I).

**Separation of decidua from trophoblast**

Haematoxylin and eosin staining and immunohistochemistry for cytokeratin demonstrated clear isolation of decidua from trophoblast by macroscopic separation in saline. The state of inflammation of the samples was also determined—each sample was rigorously examined for presence of necrosis, and lymphocyte and polymorph infiltration. These features were present to varying degrees, but there was no obvious difference (data not shown). All stained tissue sections were examined by an expert histopathologist.

**Chlamydia serology**

All decidal biopsies were screened for Chlamydia trachomatis infection and shown to be negative.

**SLPI and elafin mRNA expression in uterine decidua from intra and extrauterine pregnancies**

Elafin and SLPI mRNA were detected in the uterine decidua of each clinical group. Relative SLPI mRNA expression was greater in the uterine decidua of women with tubal pregnancy (12.37 ± 2.66) compared with the decidua from women undergoing STOP (4.8 ± 1.45) and surgical management of spontaneous abortion (5.09 ± 2.22) (Table I, Fig. 1A). However, ANOVA analysis of logged data demonstrated a statistically significant difference (overall P < 0.0185, Tukey post hoc analysis P < 0.05) between the tubal pregnancy and spontaneous abortion groups only. Elafin mRNA expression also was higher in the decidua from women with tubal pregnancy (73.57 ± 35.29) compared with those with spontaneous abortion (9.00 ± 2.53) or undergoing a STOP (4.6 ± 1.38) (Table I, Fig. 1B). However, this observation was not significant using ANOVA, or non-parametric, statistical analysis of the normal, or logged, data. This was attributed to the high variability of expression in the tubal pregnancy group. Two samples were excluded from analysis (one from a patient undergoing a STOP and the other from a patient with a first trimester spontaneous abortion) because their relative mRNA values for elafin were greater than 2SDs of the mean of the other samples.

**Immunohistochemical localization of elafin and SLPI protein in uterine decidua from intra and extrauterine pregnancies**

Elafin and SLPI protein expression and distribution were demonstrated in the uterine decidua by immunohistochemistry (Fig. 2A–F). There was no obvious difference in elafin and SLPI protein expression pattern in any of the clinical groups. SLPI protein expression was demonstrated in the epithelium (Fig. 2A and B) and decidual leukocytes (Fig. 2C). SLPI protein expression was particularly strong in the cytoplasm of the glandular epithelium (Fig. 2B). Elafin protein expression was restricted to the decidual leukocyte populations (Fig. 2E and F). However, in some cases, there was evidence of membranous elafin protein expression in the epithelial glands (Fig. 2D). The negative controls for elafin and SLPI immunorexpression showed no evidence of positive staining (Fig. 2G and H, respectively). All stained tissue sections underwent qualitative analysis by an expert pathologist. No quantitative analysis was performed due to the poor reproducibility and inter-observer agreement of immunohistochemistry scoring (Taylor, 1994).

**Discussion**

To our knowledge, we are the first to report differences in gene expression of natural antimicrobial peptides (SLPI and elafin) in the uterine decidua collected from women with tubal...
pregnancy compared with those with an intrauterine gestation. This study demonstrates that SLPI mRNA expression is significantly higher (P < 0.05) in the uterine decidua of women with a tubal compared with a failed intrauterine gestation (spontaneous abortion). Previous studies have only compared the cellular composition of the uterine decidua, with particular attention to the leukocyte populations, and found no obvious distinction between extra and intrauterine pregnancies (Earl et al., 1987; Stewart-Akers et al., 1997).

The most obvious difference in the decidua between the tubal pregnancy and intrauterine groups is the absence of trophoblast. There is no published data to our knowledge to suggest that SLPI or elafin expression is regulated by trophoblast signals. Nevertheless, recent in vitro studies, using a functional genomics approach, have shown that conditioned media from trophoblasts alter the local immune environment of the decidua to facilitate embryo implantation by causing a significant induction of proinflammatory cytokines and cells (Hess et al., 2007). This provides an interesting potential mechanism for the changes observed in our study.

Alternatively, if the uterine environment truly reflects that of the fallopian tube, the increased expression of SLPI in the uterine decidua may simply be a consequence of extratubal implantation. In the fallopian tube, it is essential that the normal oviductal epithelium has the capacity to recognize and respond to ascending pathogens while simultaneously avoiding a state of unnecessary inflammation that might disrupt the epithelial barrier. Foreign pathogens are recognized by pattern recognition receptors present on the oviduct cells, such as the membraneous TLRs (Darville et al., 2003; Pioli et al., 2004). Binding initiates a signalling cascade, leading to NF-kappaB activation and the subsequent production of pro-inflammatory and immunoregulatory cytokines, chemo-kines and co-stimulatory molecules (e.g. interleukin-1, tumour necrosis factor) (Bowie and O’Neill, 2000). NF-kappaB may indirectly increase SLPI expression via the up-regulation of these inflammatory molecules (King et al., 2002). There is often no evidence of acute inflammation around a tubal implantation site (Kutluay et al., 1994). In ectopic pregnancy, tubal expression of SLPI during embryo implantation may reduce the inflammatory response and subsequent early tubal damage by inhibiting protease actions. SLPI has been shown to reduce tissue damage in models of emphysema and fibrosis of the lung, and studies have shown reduced levels of SLPI in the gastric mucosa of patients with Helicobacter pylori induced gastritis (Mulligan et al., 1993; Rudolphus et al., 1993; Mitsuhashi et al., 1996; Hritz et al., 2006; Wex et al., 2004a,b, 2006). The elevated levels of SLPI in the event of an ectopic implantation may serve as an explanation for the lack of acute inflammation observed.

However, it is also possible that the increased expression of SLPI may predispose to, rather than be a consequence of, tubal pregnancy. Chlamydia trachomatis, the most common bacterial sexually transmitted infection in the UK, has been shown to be the principal risk factor for tubal pregnancy (Farquhar, 2005; Cassell et al., 2006). Nevertheless, although it has been suggested that Chlamydial infection is one of the major causes of tubal damage (Odland et al., 1993), the pathogenic events that lead from Chlamydial infection to tubal pregnancy are unclear. Although we have shown that our samples showed no evidence of current infection, it is difficult to demonstrate past infection with current assays. Our own in vitro studies have demonstrated that Chlamydia induces expression of SLPI in endometrial epithelium (Horne et al., unpublished data) and trophoblast (Wheelhouse et al., in preparation). It is possible that Chlamydia acts via NF-kappaB by way of the above signalling cascade, and that persistent, or repeated, infection causes atypical expression of SLPI in the

Table 1. Data from women with tubal or intrauterine pregnancy who underwent biopsy of uterine decidua for analysis of SLPI and elafin expression.

<table>
<thead>
<tr>
<th></th>
<th>STOP*</th>
<th>Spontaneous abortion*</th>
<th>Tubal*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation (days)**</td>
<td>58.75 ± 2.49</td>
<td>57.66 ± 7.81</td>
<td>58.09 ± 8.28</td>
</tr>
<tr>
<td>Serum progesterone (ng/l)**</td>
<td>66.01 ± 10.31</td>
<td>33.92 ± 14.99</td>
<td>58.53 ± 47.22</td>
</tr>
<tr>
<td>SLPI ***</td>
<td>4.6 ± 1.45</td>
<td>5.09 ± 2.22</td>
<td>12.37 ± 2.66</td>
</tr>
<tr>
<td>Elafin***</td>
<td>4.6 ± 1.38</td>
<td>9.00 ± 2.53</td>
<td>73.57 ± 35.29</td>
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</tbody>
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*One sample discarded for elafin analysis.
**Mean ± SD.
***mRNA expression relative to control (mean ± SEM).
STOP, surgical termination of pregnancy.

Figure 1: Graphical representation (box and whisker plot) of relative SLPI (A) and elafin (B) mRNA expression in uterine decidua from women undergoing STOP, surgical management of spontaneous abortion and surgical management of ectopic (tubal) pregnancy (*P < 0.05 Tukey post hoc analysis).
The demonstration of an altered level of SLPI mRNA expression in women with ectopic compared with intrauterine pregnancies contributes further to our current knowledge of embryo implantation. Although immunohistochemistry demonstrated no obvious difference in the pattern of distribution or level of elafin and SLPI protein expression, we acknowledge the difficulty in accurately assessing secretory proteins using this technique and are aware that further quantitative protein analysis and functional studies are required.

Author’s Role

D.M.D.: laboratory work, data analysis and manuscript preparation.
J.M.S.: data analysis and manuscript preparation.
H.O.D.C.: data analysis and manuscript preparation.
A.R.W.: data analysis and manuscript preparation.
W.Y.T.: laboratory work.
A.E.K.: data analysis and manuscript preparation.
A.W.H.: data analysis and manuscript preparation.

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