Secretory leukocyte protease inhibitor (SLPI) concentrations in cervical mucus of women with normal menstrual cycle

Akihiro Moriyama¹, Koichiro Shimoya¹²⁴, Isao Ogata¹, Tadashi Kimura¹, Takafumi Nakamura¹, Hiroko Wada¹, Kazutomo Ohashi¹, Chihiro Azuma¹, Fumitaka Saji³, and Yuji Murata¹

¹Department of Obstetrics and Gynecology, Faculty of Medicine, Osaka University, 2–2 Yamada-oka, Suita City, Osaka 565-0871, ²Department of Obstetrics and Gynecology and ³Department of Gynecology, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1–3–3 Nakamichi, Higashinari-ku, Osaka 537-0025, Japan. e-mail: shimoya@gyne.med.osaka-u.ac.jp

Secretary leukocyte protease inhibitor (SLPI) is a potent inhibitor of human leukocyte elastase. SLPI transcripts in the cervical tissue were detected during the menstrual cycle by reverse transcription–polymerase chain reaction (RT–PCR). Western blot analysis revealed that the intensity of SLPI protein in cervical tissue in the ovulatory phase was stronger than in other phases. Immunohistochemistry using an anti-SLPI polyclonal antibody revealed positive staining in the epithelial cells of the endocervix. Western blot analysis also revealed that SLPI protein was present in the cervical mucus. Again the intensity of SLPI protein in the ovulatory phase was stronger than that in the follicular phase. The SLPI concentrations and SLPI/elastase ratios in the cervical mucus of women in the ovulatory phase were significantly higher than in the follicular and luteal phases. The SLPI and elastase concentrations in the cervical mucus were positively correlated. No significant difference was found in the SLPI serum concentrations of women during the menstrual cycle. These results suggest that production of SLPI from cervical epithelial cells during the ovulatory phase may be important for protection from the effects of elastase.

Key words: cervical mucus/ovulation/secretory leukocyte protease inhibitor (SLPI)

Introduction

It is important for the investigation of infertility to evaluate cervical factor and sperm–cervical mucus interactions. Abnormalities of the cervix and its secretion are responsible for infertility in ~5–10% of infertile women (Moghissi, 1995). Cervical mucus is a complex secretion produced constantly by the secretory cells of the endocervix. The cervix produces mucus at the rate of 20–60 mg/day in normal women of reproductive age. During the midcycle, the amount increases 10–20-fold and may reach up to 700 mg/day (Moghissi and Syner, 1976). Cyclic variations in the amount, physical properties, and chemical content of the cervical mucus constituents have been reported. From the termination of menstruation to the time of ovulation, viscosity and flow elasticity progressively decrease and spinbarkeit increases. After ovulation and during the luteal phase, spinbarkeit decreases and flow elasticity and viscosity markedly increase (Moghissi, 1995). Cervical mucus has bacteriostatic and bactericidal properties against certain strains of bacteria. Various bacteria are unable to migrate in a capillary tube filled with ovulatory cervical mucus. Bactericidal activity of the human cervical mucus is present during all phases of the menstrual cycle but is least pronounced at ovulation (Enhorning et al., 1970).

Pooled human cervical mucus contains ~1–3% protein in two basic forms, soluble proteins and mucin (Moghissi, 1995). The major components of soluble proteins are albumin and gamma globulin. Cyclic variations in the amounts of several proteins in cervical mucus have been described. In general, there appears to be a pre-ovulatory decrease and a post-ovulatory increase in the amounts of albumin, α1-antitrypsin, and immunoglobulins (Ig) (Schumacher and Pearl 1968; Schumacher, 1970). Mucins comprise 45% of proteins in the cervical mucus. Mucin plays an important role in sperm transport. The secretion of cervical mucus is regulated by ovarian hormones. Oestrogen stimulates the production of copious amounts of watery mucus, whereas progesterone inhibits the secretory activity of cervical epithelial cells (Moghissi, 1995). The change of cervical mucus may also influence sperm penetrability, nutrition, and survival. Pre-ovulatory mucus is most receptive to sperm penetration (Moghissi et al., 1972).

Secretory leukocyte protease inhibitor (SLPI) is a potent inhibitor of human leukocyte elastase, human cathepsin G, and human trypsin (Thompson and Ohlsson, 1986). The concentrations of SLPI in biological samples have been monitored to correlate these concentrations with pathological conditions (Kida et al., 1992; Kouchi et al., 1993; Sluis et al., 1994). SLPI is found in various fluids, including parotid secretions (Thompson and Ohlsson, 1986), bronchial, nasal (Fryksmark et al., 1989), cervical mucus (Casslen et al., 1981; Helmig et al., 1995), and seminal plasma (Ohlsson et al., 1995; Moriyama et al., 1998). We have reported the beneficial...
effect of SLPI on sperm motility damaged by elastase. No study of the relationship between SLPI and elastase in cervical mucus during menstrual cycle has been reported. Evaluations of SLPI concentrations and elastase titres in the cervical mucus and SLPI gene transcript in the cervical tissue are necessary. In this study, SLPI protein was detected in the cervical mucus and cervical tissue by Western blot analysis. SLPI and elastase were quantified in cervical mucus from women with normal menstrual cycles by an enzyme-linked immunosorbent assay (ELISA). SLPI transcripts were also demonstrated in the cervical tissue by reverse transcription–polymerase chain reaction (RT–PCR) and SLPI producing cells by an immunohistochemical method.

Materials and methods

Samples
A total of 11 non-pregnant women (aged 24–35 years) with normal ovulatory cycles confirmed by basal body temperature and vaginal sonography were recruited for this study, and informed consent was obtained. None of the subjects had a history of venereal infection, and women infected with bacteria or Chlamydia were excluded. Ovulation was confirmed by a urinary luteinizing hormone (LH) test, transvaginal sonography, mid-luteal phase progesterone and basal body temperature charts. Cervical secretion samples (n = 155) were collected with sterile Dacron swabs as previously described (Kanai et al., 1997); the Dacron swab was used to aspirate 150 µl of cervical mucus. The samples were diluted with saline for 30 min at room temperature. Debris and cells were removed by centrifugation at 1000 g for 15 min, and the supernatants were stored at –20°C until titration to determine the SLPI and elastase concentrations. At the time of collection of cervical mucus, serum was also collected and stored at –80°C until titration to determine the SLPI and elastase concentrations. At the time of collection of cervical mucus, serum was also collected and stored at –80°C until titration to determine the SLPI and elastase concentrations. For use in a Western blot analysis, some of the cervical mucus samples in the follicular phase and the ovulatory phase were obtained by aspiration from the endocervix with a long tuberculin syringe. It was impossible to collect cervical mucus in the luteal phase by aspiration with a long tuberculin syringe. Surgical specimens of the cervix were obtained at a hysterec-
SLPI polyclonal antibody. The primary antibody was used at a final concentration of 1.0 μg/ml. The SLPI immunoreactivity was visualized using an enhanced chemiluminescence (ECL) Western blotting analysis system (Amerham, Aylesbury, UK).

Immunohistochemical staining of SLPI in the uterine cervix
To determine the localization of SLPI in the uterine cervix, we performed immunohistochemical staining using an avidin–biotin peroxidase complex method kit (OminiTags Universal Streptavidin/Biotin Affinity Immunostaining Systems, Lipshaw, Pittsburgh, PA, USA). Fresh frozen sections of the cervix were bleached in 0.3% hydrogen peroxide to block endogenous peroxidase and covered with 2% goat IgG to minimize non-specific binding. The appropriately diluted goat polyclonal anti-SLPI antibody (R&D Systems, Minneapolis, MN, USA) or control goat IgG for the control was applied at room temperature and left for 1 h. After rinsing with phosphate-buffered saline solution, the sections were further incubated for 30 min with biotin-labelled goat anti-mouse immunoglobulin G, followed by the addition of avidin–peroxidase complex at 4°C. Peroxidase activity in the sections was visualized with 0.1% 3,3-diaminobenzididine-tetrahydrochloride containing 0.02% hydrogen peroxide in 0.1 mol/l Tris buffer (pH 7.2). The slides were counterstained with Mayer’s haematoxylin.

Determination of SLPI in the cervical mucus by ELISA
To determine concentrations of SLPI in the cervical mucus, ELISA kits utilizing a monoclonal antibody specific for SLPI (R&D Systems, Minneapolis, MN, USA) were used. The SLPI concentration detection limit of this kit was 62.5 pg/ml. No cross-reactivity with cytokines, growth factors, elastase, trypsin, and chymotrypsin could be found in this kit. The intra-assay variation of the SLPI kit was 4.2–8.0%, and its inter-assay variation was 4.9–8.0%.

Determination of elastase titre in the cervical mucus by ELISA
To measure the titres of elastase in the cervical mucus, ELISA kits specific for elastase (Merck, Darmstadt, Germany) were used. Cervical mucus titres of elastase which the kit detected were >1.0 µg/l. The intra-assay and inter-assay variation of the elastase kit were 2.7–5.2% and 4.9–9.5% respectively.

Statistical analysis
Statistical analyses of SLPI, elastase concentrations, and the SLPI/elastase ratio in cervical mucus were conducted using a non-parametric test; P < 0.05 was considered to be statistically significant. The correlation between SLPI and elastase in cervical mucus was analysed by simple linear regression.

Results
RT–PCR was performed to determine the expression of the SLPI gene in the cervical tissue during the menstrual cycle. Figure 1 shows that SLPI transcripts were present in the cervical tissue during the menstrual cycle. As shown in Figure 2, the Western blot analysis detected SLPI protein in the cervical tissue as a 12 kDa band. The intensity of SLPI in the cervical tissue in the ovulatory phase was stronger than that in both the follicular and luteal phases. To identify the origin of this large amount of SLPI, we performed immunohistochemical staining of sections of the uterine cervix in the ovulatory phase, using an anti-SLPI polyclonal antibody. The cytoplasm of columnar epithelial cells in the endocervical glands, the subepithelial layers, and cervical mucus were intensely stained (Figure 3). To examine SLPI protein in the cervical mucus, we performed a Western Blot analysis. As shown in Figure 4, SLPI protein was detected as a 12 kDa band in the cervical mucus. The slightly lower molecular weight band seen in Figures 2 and 4 is thought to be a degradation product of SLPI. The intensity of SLPI protein in the ovulatory phase was stronger than that in the follicular phase. We determined the SLPI concentrations in the cervical mucus with a specific ELISA for SLPI. Figure 5 shows the SLPI concentrations in the cervical mucus of women during the menstrual cycle. The SLPI titres, elastase concentrations and SLPI/elastase ratio in the cervical mucus of each phase during the menstrual cycle were calculated by the average of those of each phase. The SLPI titres of cervical mucus in the follicular phase were in the range 7–667 ng/ml (median: 188 ng/ml) and those in the luteal phase in the range 14–520 ng/ml (median: 110 ng/ml), while those in the ovulatory phase were...
in the range 352–1040 ng/ml (median: 880 ng/ml). There is a significant difference between SLPI concentrations between the three phases \((P < 0.0001)\). The elastase concentrations of cervical mucus in the follicular phase were in the range 7–667 ng/ml (median: 188 ng/ml), those in the luteal phase were in the range 14–520 ng/ml (median: 110 ng/ml) and those in the ovulatory phase were in the range 352–1040 ng/ml (median: 880 ng/ml). There was a significant difference between SLPI concentrations in these three phases \((P < 0.0001)\).
elastase ratios of these three phases was significant (range 0.006–0.230 (median: 0.036). The difference in the SLPI/elastase ratio in the follicular phase was in the range 0.005–0.279 (median: 0.055) and that in the luteal phase was in the range 0.006–0.230 (median: 0.036). The difference in the SLPI/elastase ratios of these three phases was significant ($P < 0.001$).

**Figure 6.** Change in secretory leukocyte protease inhibitor (SLPI)/elastase ratio in the cervical mucus during normal menstrual cycles ($n = 11$). The mean SLPI/elastase ratio in the cervical mucus in the ovulatory phase was in the range 0.079–0.462 (median: 0.149), that in the follicular phase was in the range 0.005–0.279 (median: 0.055) and that in the luteal phase was in the range 0.006–0.230 (median: 0.036). The difference in the SLPI/elastase ratios of these three phases was significant ($P < 0.001$).

**Table I.** Concentrations of secretory leukocyte protease inhibitor (SLPI) during the menstrual cycle

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Range (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular phase</td>
<td>36.8</td>
<td>26.2–52.2</td>
</tr>
<tr>
<td>Ovulatory phase</td>
<td>34.7</td>
<td>26.3–49.9</td>
</tr>
<tr>
<td>Luteal phase</td>
<td>42.0</td>
<td>26.3–48.3</td>
</tr>
</tbody>
</table>

significant difference of elastase titres in the cervical mucus among the three phases ($P = 0.09$) was observed. As shown in Figure 6, the SLPI/elastase ratio in the cervical mucus in the ovulatory phase was in the range from 0.079–0.462 (median: 0.149), while that in the follicular phase was in the range 0.005–0.279 (median: 0.055) and that in the luteal phase was in the range 0.006–0.230 (median: 0.036). The difference in the SLPI/elastase ratios of these three phases was significant ($P < 0.001$). We also examined the SLPI concentrations in the serum of the subjects. As shown in Table I, there was no significant difference in the SLPI serum concentrations during the menstrual cycle. To examine the correlation between SLPI and elastase concentrations in cervical mucus, a simple linear analysis was performed. The SLPI and elastase concentrations in the cervical mucus were positively correlated ($y = 4.4x + 2220$, $r = 0.47$, $P < 0.0001$).

**Discussion**

In the present study, it was found that the SLPI gene is expressed during the menstrual cycle. No significant difference in SLPI gene expression was detected during the menstrual cycle by RT–PCR. However, there was a difference between the SLPI protein concentrations in the cervical tissue during the menstrual cycle. Western blot analysis revealed that the intensity of SLPI in the cervical tissue in the ovulatory phase was stronger than that in both the follicular and luteal phases. SLPI gene 5' flanking region has a TATA box and multiple potential nuclear activator protein-1 (AP-1) binding sites, which are capable of mediating a specific response to induction by phorbol esters. However the mechanisms controlling SLPI gene expression in vivo are unknown (Abe et al., 1991). An immunohistochemical analysis using anti-SLPI polyclonal antibody showed that the columnar epithelial cells of the endocervical glands were intensely stained, indicating that these epithelial cells were the main source of SLPI in the cervical mucus. The results presented here of an immunohistochemical analysis are consistent with previous findings (Casslen et al., 1981).

The cervical mucus contains various kinds of enzymes, e.g. amylase, alkaline phosphatase, esterase, aminopeptidase, lactate dehydrogenase, and peroxidase. These enzymes show a marked pre-ovulatory decrease and post-ovulatory rise in response to progesterone in the luteal phase. It has been suggested that assays of some of the enzymes that exhibit a pre-ovulatory decline and post-ovulatory rise may be used to predict or detect ovulation (Moghissi, 1995). The change of SLPI concentrations in the cervical mucus of women during the menstrual cycle was observed in the present study. The mean SLPI titres of women in the ovulatory phase were higher than those of women in both the follicular phase and the luteal phase. However, there is no difference in the serum SLPI of the women during the menstrual cycle. These results indicate that the change in SLPI concentrations in cervical mucus is a localized reaction in the menstrual cycle. The secretion of cervical mucus is regulated by ovarian hormones. Oestrogen stimulates the production of copious amounts of watery mucus, whereas progesterone inhibits the secretory activity of cervical epithelial cells. SLPI production by the cervical tissue might be regulated by ovarian hormones. It was also reported that antileukoprotease concentrations in luteal phase cervical mucus were higher than those of the follicular and ovulatory phases (Casslen et al., 1981), in contrast to the results of this study. Casslen et al. reported that the latter part of the menstrual cycle is a period when numerous leukocytes are found in the uterus, a situation which presumably presents a significant task for inhibitors like SLPI. The discrepancy might be due to the different methods used to obtain cervical mucus and the different methods used to determine the protein concentrations. Further investigations are necessary to explain the discrepancy in results.

Cervical mucus has bacteriostatic and bacteriocidal properties. Elastase is a protease which is produced by leukocytes in the cervix (Moghissi, 1995). Cervical mucus and semen contain large amounts of elastase (Wolff and Anderson, 1988; Shimoya et al., 1993). However, no significant difference of elastase titres in the cervical mucus during the menstrual cycle was observed here. SLPI is an inhibitor of proteases such as leukocyte elastase (Ohlsson et al., 1995). In the present study, the mean SLPI titre and SLPI/elastase ratio of the women in the ovulatory phase were higher than those of the women in the follicular or luteal phases. The up-regulation of SLPI plays...
a defensive role in the epithelial surface of inflammatory lung diseases (Abbinante et al., 1993). SLPI might protect the cervical epithelium from the leukocyte protease of cervical mucus and semen.

The sperm–cervical mucus interaction is an important factor for fertilization. Cyclic alterations in the concentrations of cervical mucus may also influence sperm penetrability, nutrition, and survival. Pre-ovulatory mucus is most receptive to sperm penetration (Moghissi and Syner, 1976). It is usually inhibited within 1–2 days after ovulation but may persist to a lesser degree for a longer period (Moghissi, 1995). It has previously been demonstrated that SLPI recovered sperm motility reduced by elastase which is contained in the seminal plasma (Moriyama et al., 1998). In human cervical mucus, motile spermatozoa have been found 2–8 days after coitus (Moghissi, 1995). Because the cervical mucus in the ovulatory phase contains high amounts of SLPI, this molecule might have an important effect on the sperm penetrability of human cervical mucus. Further investigations are necessary to examine the relationship between SLPI concentrations and cervical factors in infertility.

Acknowledgements
This work was supported in part by Grants-in-Aid for Scientific research (Nos. 20151061, 30203897, 50294062, 70283786, 80301266 and 90093478) from the Ministry of Education, Science, and Culture of Japan (Tokyo, Japan).

References

Received on November 26, 1998; accepted on April 9, 1999