Cervical Expression of Elafin and SLPI in Pregnancy and Their Association With Preterm Labor

Nao Itaoka[1], Takeshi Nagamatsu[1], Danny J. Schust[2], Mayuko Ichikawa[1], Seisuke Sayama[1], Yuki Iwasawa-Kawai[1], Kei Kawana[1], Takahiro Yamashita[1], Yutaka Osuga[1], Tomoyuki Fujii[1]

[1]Department of Obstetrics and Gynecology, Faculty of Medicine, The University of Tokyo, Tokyo, Japan;
[2]Department of Obstetrics, Gynecology and Women's Health, University of Missouri, Columbia, MO, USA

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Correspondence
Takeshi Nagamatsu, Department of Obstetrics and Gynecology, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: tnag-tky@umin.ac.jp

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Problem
Elafin and secretory leukocyte peptidase inhibitor (SLPI) are unique among antimicrobial peptides (AMPs). This study aimed to determine the expression levels of these AMPs at the cervix during pregnancy and to investigate their association with preterm labor.

Method of study
Cervical epithelial cells were swabbed from normal pregnant women to evaluate the physiological expression of elafin and SLPI. Cross-sectional analysis was conducted to compare cervical expression levels for SLPI and elafin among three women's groups, controls (n = 26), women with threatened preterm labor who delivered at term (t-TPL, n = 23) and TPL who ended in preterm labor (p-TPL, n = 19).

Results
Elafin and SLPI proteins were detected in the squamous and glandular cells of the cervix. Cervical SLPI expression levels increased over the course of pregnancy, whereas elafin levels remained unchanged. Cervical mRNA expression levels of elafin and SLPI were significantly higher in p-TPL compared with t-TPL and control groups.

Conclusion
Constitutive expression of elafin and SLPI in cervical cells during pregnancy suggests their essential roles in local tissue homeostasis and immune defense. The elevations in cervical elafin and SLPI expression in the women with preterm delivery might reflect the local response to the pathogen invasion into the cervix preceding preterm labor.

Introduction

Antimicrobial peptides (AMPs) play essential roles in the modulation of infection and inflammation by the innate immune system. AMPs have broad-spectrum antimicrobial activities against bacteria, fungi, and some viruses.[1,2] Elafin and secretory leukocyte peptidase inhibitor (SLPI) are categorized as AMPs. They are expressed in a variety of cell types including epithelial cells and inflammatory cells.[3–7] Similar to other AMPs, their main antimicrobial activities are thought they involve destabilization of microbial membranes by means of their amphipathic structure and cationic charge.[8,9] In addition to their antimicrobial activities, elafin and SLPI share antiprotease properties. They are classified within the whey acid protein (WAP) family because they possess WAP motifs, which are known to function as inhibitors of serine proteases, such as neutrophil elastase. Elafin and SLPI share 40% homology, with SLPI holding

two WAP domains and elafin holding one WAP domain.[8–10] These protease inhibitors prevent tissue destruction caused by excessive protease activities induced upon inflammation. The anti-inflammatory properties of these two AMPs include potent inhibition of inflammation-induced NF-κB transcription.[8] Taken together, the molecular properties of elafin and SLPI promote the abrogation of infectious pathogens while minimizing extensive inflammatory tissue damage.

Preterm labor is a significant clinical problem in obstetrics that causes a high degree of morbidity in mothers and both morbidity and mortality in neonates. Approximately 30–50% of preterm labors are associated with intrauterine infection.[11,12] Infection is more commonly a cause for preterm labor when delivery occurs at <30 weeks as opposed to preterm deliveries closer to term.[13] During pregnancy, the uterine cervix typically acts as an effective barrier against external pathogens. When this protection system fails, the local inflammatory response that develops to fight off infectious microorganisms can result in cervical remodeling and premature labor and preterm delivery. Although roles for elafin and SLPI at the genital tract have been described,[6,14] little is known about their involvement in physiological and pathological processes during pregnancy. This study aimed to determine the expression levels of those protease inhibitors at the level of the cervix in human pregnancy and to investigate their relevance to the pathology of preterm labor.

Materials and methods

Sample Collection

This study was conducted under the approval of institutional review board in the University of Tokyo. Patients' consent was given upon the collection of clinical samples. All the women enrolled in this analysis are Japanese, and their demographic data are as follows: age: 32.8 ± 4.6 years (mean ± S.D.), parity: nulliparous 57% and multiparous 43%, rate of women with smoking habit during pregnancy: 2.4%. The women with medical complication other than threatened preterm birth were excluded from this study.

Aiming to investigate physiological shift in elafin and SLPI expression during pregnancy, cervical cells were collected from 144 pregnant women at different stages of normal gestation: 36 subjects in the first trimester, 41 subjects in the second trimester, 36 subjects in the third trimester, and 31 postpartum subjects. Cervical cells were collected during speculum examination and prior to digital examination and transvaginal ultrasound assessment by inserting the tip of a sterile swab into the cervical canal and gently rolling it. Swabs were immediately smeared onto a glass slide, and the slide was sprayed with Cytology Fixative Spray (Japan medi port, Sapporo, Japan) for immunocytochemical study. For mRNA expression analysis, similarly collected swabs were stirred for 5 s in 2 mL of extraction buffer (FARB buffer; Blood/Cultured Cell Total RNA Purification Mini Kit; FAVOGEN, Ping-Tung, Taiwan).

For preterm birth analyses, cervical cell samples were taken from 42 women with singleton pregnancies who were admitted for bed rest in the hospital with the diagnosis of threatened preterm labor (TPL). The cervical cell samples were collected soon after admission, when the symptoms associated with TPL were confirmed. The gestational ages at cervical sampling of women in the TPL group ranged from 20 to 35 weeks. Gestational age-matched controls for the preterm birth cohort were selected from the normal pregnant control subjects involved in the 'physiological shift' study described above (n = 26).

In the analysis for the alterations in cervical elafin and SLPI expression depending on pregnancy outcome, women with TPL were further divided into two groups, p-TPL: women who delivered prematurely (n = 19) and t-TPL: women who ultimately delivered after 37 weeks of gestation (n = 23). Both groups were clinically managed under the same treatment protocol during hospitalization.

Diagnosis and Clinical Management of TPL

The diagnosis of TPL requiring hospitalization was made when at least one of the following symptoms was observed: (i) The cervical length measured by transvaginal ultrasonography was shorter than 25 mm at <28 weeks of gestation, and (ii) uterine contractions resulted in dilatation of the cervix at <35 weeks of gestation. Women with premature rupture of membrane were excluded from this analysis. Therapeutic management in the hospital included (i) intravenous tocolytic agents when uterine contractions were detected on cardiotocography, and (ii) administration of antibiotics to women with elevations in peripheral white blood cell count and/or serum C-reactive protein (CRP).

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Immunocytochemistry of Uterine Cervical Cells

Elafin and SLPI protein levels in cervical cells were examined by immunocytochemistry. Cervical cell smears from 22 pregnant women without complications were assessed: seven women in the first trimester, eight women in the second trimester, and seven women in the third trimester. Endogenous peroxidase activity and non-specific antibody binding were blocked with peroxidase-blocking solution (Dako Japan, Tokyo, Japan) and protein block serum free (Dako Japan), respectively. Fixed cell smears were incubated overnight with a primary antibody against elafin (Atlas Antibodies, Stockholm, Sweden; HPA017737, diluted to 1:400) or SLPI (Hycult biotech, Uden, the Netherlands; HM2037, diluted to 1:50). The dilution optimization of the primary antibodies was performed by examining dilution series in the preliminary experiments using positive control specimen of HeLa cells. Rabbit immunoglobulin fraction (Dako Japan) or normal mouse IgG1 (Dako Japan) was used as a negative control for primary antibodies, and their concentrations were matched to those of the respective primary antibody. Specimens were then incubated with an appropriate secondary antibody (Envision-plus Dual Link System-HRP; Dako Japan). DAB substrate (Dako Japan) was used to visualize specific staining. The cells were counterstained with hematoxylin.

Extraction of mRNA from Cervical Cell Samples

Cells collected in the extraction buffer were immediately processed for mRNA extraction using a Blood/Cultured Cell Total RNA Purification Mini Kit (FAVORGEN, Ping-Tung, Taiwan). In the extraction procedure, genomic DNA was removed using DNAse I (0.5 U/μL, Life Technologies Japan, Tokyo, Japan) according to the manufacturer's protocol. mRNA concentration and quality were assessed using an Epoch Micro-Volume Spectrophotometer System (Bio-Tek, Winooski, VT, USA). The mRNA samples were reverse transcribed into cDNA using ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) for subsequent real-time PCR analysis.

Assessment of mRNA Expression for Elafin and SLPI

Cervical cell mRNA expressions of elafin and SLPI were measured using real-time PCR (Light Cycler; Roche Diagnostics K.K., Tokyo, Japan). Five microlitre of the sample cDNA (0.5 ng/μL) and a pair of specific primers were mixed with Light Cycler 480 SYBR Green I Master (Roche Diagnostics K.K.), making a total PCR mixture volume of 20 μL. The expression level of β-actin was used to normalize the mRNA amount in each sample. The sequences of the primers were as follows: (i) elafin: forward primer, CGTGGTGGTGTTCCTCATC; reverse primer, GACCTTTGACTGGCTCTTGC; product size, 179 bp, (ii) SLPI: forward primer, GGATGGCCAGTGCAAGC GTGA; reverse primer, GCCTGCTGTGTGCCAAGC CT; product size, 199 bp, and (iii) β-actin: forward primer, CATGTACGTTGCTATCCAGGC; reverse primer, CTCCTTAATGTCACGCACGAT; product size, 250 bp. The PCR cycle protocol consisted of pre-incubation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 10 s, annealing at 63°C for 10 s for elafin (65°C for 10 s for SLPI), and extension at 72°C for 7 s for elafin (72°C for 9 s for SLPI), followed by melting curve analysis to confirm that the target amplicons were detected. The PCR product inserted into pCRII-TOPO plasmid vector was subcloned using TOPO TA cloning kit (Life Technologies). The accordance of nucleotide sequence with the previously reported one was confirmed by sequencing analysis.

Statistical Analysis

Statistical analysis was carried out with JMP PRO 9.0 software (SAS institute Japan, Tokyo, Japan). The evaluation of staining score data was performed using the Mann–Whitney test. In the multiple comparisons of elafin and SLPI mRNA expression levels among TPL and control groups, Kruskal–Wallis nonparametric testing, followed by a *post hoc* analysis using the Mann–Whitney test with Bonferroni correction, was performed. All the data were presented as mean ± S.D. $P < 0.05$ was regarded as statistically significant.

Results

Production of Elafin and SLPI in Uterine Cervical Cells

Production of elafin and SLPI in the cells composing the uterine cervix was evaluated using immunocytochemistry ($n = 22$). The main components of the collected specimens included squamous cells (SCs) and glandular cells (GCs). The proportion of the two

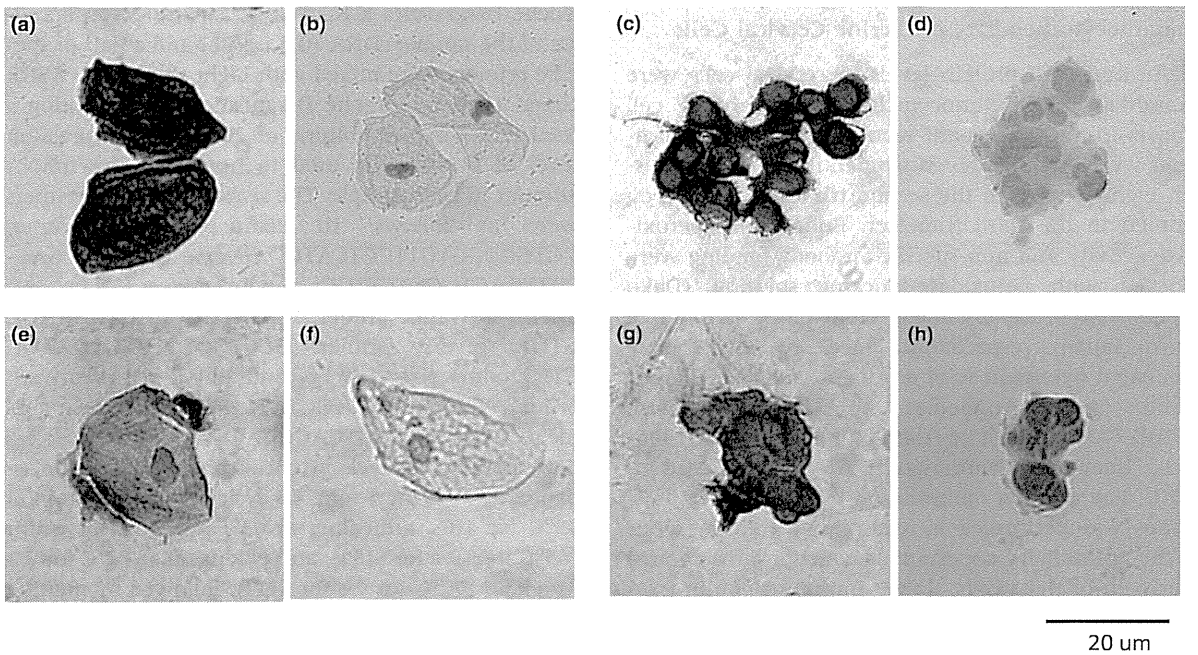

Fig. 1 *Expression of elafin and secretory leukocyte peptidase inhibitor (SLPI) protein in uterine cervical cells.* Immunocytochemistry was carried out to examine the expression of elafin and SLPI in uterine cervical cells during pregnancy. Cells collected by gently swabbing the cervical canal were immediately smeared on a slide glass. Representative images of 22 specimens from different individuals are shown. Squamous cells: a, b, e, and f. Glandular cells: c, d, g, and h. Staining for elafin is depicted by brown coloration: a and c. Staining for SLPI is depicted by brown coloration: e and g. b, d, f, and h: negative controls corresponding to the images a, c, e, and g, respectively.

cell types was approximately 4:1 (SCs:GSs), regardless of the gestational age at the time of sampling. The cell types could be easily discriminated by their characteristic morphologies. Cervical SCs were approximately 20–30 μm in diameter and polygonal in shape and exhibited a low nucleus to cytoplasm ratio (Fig. 1a,b,e,f). Cervical GCs were recognized as round cells that were typically present in clusters. At approximately 8–10 μм, GC cell size was smaller than that of SCs, and GC nuclear to cytoplasmic ratio was much higher (Fig. 1c,d,g,h). Both SCs and GCs stained positive for elafin (Fig. 1a,c) and SLPI (Fig. 1e,g) proteins. No staining was present in negative controls using non-specific immunoglobulin (Fig. 1b,d,f,h).

Physiological Shifts in Elafin and SLPI mRNA Expression Over the Course of Gestation and Postpartum

Real-time PCR analysis to examine mRNA expression levels in cervical cell samples revealed that elafin mRNA was detectable throughout gestation and the mean expression levels were unchanged over the course of pregnancy (Fig. 2a). In contrast, the expression of SLPI mRNA was significantly higher in the second and third trimester when compared to that in the first trimester (Fig. 2b). Both elafin and SLPI mRNA expression levels were remarkably high in samples from women in the postpartum period (Fig. 2a,b).

Alterations in Cervical Elafin and SLPI mRNA Expression in Women with Preterm Labor

Aiming to investigate a potential role for elafin and SLPI in the pathophysiology of preterm birth, cervical mRNA expression levels were evaluated in women with pregnancies complicated by TPL (n = 42). Women with TPL were further divided into two groups depending on pregnancy outcomes, p-TPL (n = 19) and t-TPL (n = 23), as defined in 'Materials and Methods'. Cervical cell samples (n = 26) from women with uncomplicated pregnancies were included as controls. There were no statistical differences in gestational age at the time of

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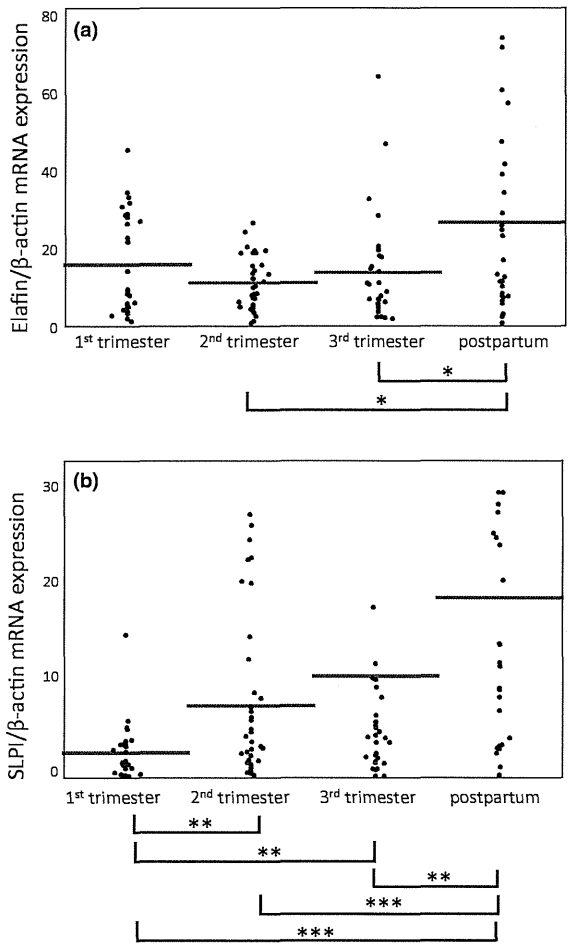

Fig. 2 Expression of elafin and secretory leukocyte peptidase inhibitor (SLPI) mRNA in uterine cervical cells in normal pregnancy. The mRNA expression levels of elafin and SLPI in uterine cervical cells isolated from normal pregnant women were analyzed using real-time RT-PCR. Cell samples were collected in the first trimester (n = 36), the second trimester (n = 41), the third trimester (n = 36), and the postpartum period (n = 31). Changes over the course of pregnancy were assessed. The bars in the chart depict mean mRNA levels in each gestational period. (a) Elafin mRNA expression. (b) SLPI mRNA expression. *P < 0.05, **P < 0.01, and ***P < 0.001.

cervical cell sampling among the t-TPL, p-TPL, and control groups. The gestational ages at delivery in the p-TPL group distributed widely from 23 weeks and 5 days to 36 weeks and 2 days (32 weeks and 1 day ± 28 days, mean ± S.D.), whereas the gestational ages at delivery were comparable between the t-TPL (38 weeks and 1 day ± 7 days) and control (38 weeks and 6 days ± 7 days) groups (Table I). No statistical difference was observed between t-TPL and p-TPL groups on the day of hospitalization in white blood cell (WBC) counts (t-TPB: 7630 ± 505 cells/μL, p-TPB: 8187 ± 748 cells/μL, p = 0.90) and CRP values (t-TPL: 0.29 ± 0.38 mg/dL, p-TPL: 0.51 ± 0.65 mg/dL, P = 0.57) in the peripheral blood (Table I). Pathological chorioamnionitis (CAM) was diagnosed more frequently in p-TPL group (6 cases of 18) than t-TPL groups (one case of 11), although pathological assessment for CAM was not performed in all cases.

The cervical mRNA expression levels of both elafin and SLPI were significantly higher in p-TPL group when compared with the t-TPL and normal control groups. No statistical difference was observed between t-TPL subjects and controls (Fig. 3a,b). The association of cervical elafin and SLPI mRNA expressions with the gestational ages at delivery was shown on scatter plots (Fig. 3c,d). In both of elafin and SLPI, the women with elevated expression distributed more frequently in the period less than gestational age at day 259 (37 weeks and 0 day). When focusing on the deliveries at preterm period, no apparent correlation between expression levels and the gestational period at delivery was observed in either elafin or SLPI (Fig. 3c,d).

Although the mean expression levels of elafin and SLPI were typically higher in p-TPL cases, the women with high SLPI levels were not necessarily identical to the women with high elafin levels. No definitive correlation between cervical elafin and SLPI expression could be detected in the p-TPL group.

Discussion

Using immunohistochemistry and real-time RT-PCR, we have shown that SCs and GCs collected from the uterine cervices constitutively express elafin and SLPI. Cervical expression levels for each of these antimicrobial proteases were predictably elevated in women hospitalized for preterm labor who ultimately delivered at preterm. This was in clear contrast to those hospitalized for TPL whose pregnancies were successfully extended to term.

It is well known that AMPs released at epithelial surfaces in the female genital tract play essential roles in innate immunity.[6,14] The present study revealed that elafin and SLPI, representative WAP family antiproteases with antimicrobial properties, are produced in substantial amounts in cervical epithelial cells throughout normal, uncomplicated

Table I Gestational ages at cervical cell sampling and at delivery

	Control	t-TPL	p-TPL	*P* value
Gestational age at cervical cell sampling	209 ± 33 days (29 weeks and 6 days)	207 ± 32 days (29 weeks and 4 days)	206 ± 25 days (29 weeks and 3 days)	NS
Gestational age at delivery	272 ± 7 days (38 weeks and 6 days)	267 ± 7 days (38 weeks and 1 day)	225 ± 28 days (32 weeks and 1 day)	<0.001*
Number of samples	26	23	19	

Gestational ages were shown as mean ± S.D. days, ex. Expected date of delivery corresponds to day 280. The values in the parentheses indicate the mean gestational ages in 'week and day' style.
NS, No statistical significance in the comparison between any pair out of three groups.
*The *P* values were below 0.001 in the comparison between t-TPL and p-TPL and between control and p-TPL.

pregnancy and during the postpartum period. In agreement with this study, Stock et al.[15] documented the presence of elafin in cervicovaginal secretions from pregnant women. Helmig et al.[16] detected high concentrations of SLPI in the cervical mucous plug during human pregnancy. In addition to the cervix, the presence of these AMPs has been reported in fetal membranes, amniotic fluid, and placental trophoblast cells.[17,18] This broad distribution of elafin and SLPI in gestational tissues suggests that they may play an important part in local immune-mediated prevention of invasion by infectious pathogens.

We found that SLPI mRNA expression levels were higher in the second and the third trimester when compared with the first trimester. In contrast, elafin expression levels were relatively unchanged over the course of pregnancy. Although the precise factors controlling cervical SLPI expression in normal pregnancy have not been clarified, pregnancy hormones, particularly progesterone, are likely involved. In a previous study, progesterone exposure increased SLPI, but not elafin mRNA expression in a breast epithelial cell line.[19] This effect was abrogated by antigestagens. In addition, DNA microarray analysis using the endometrium of Rhesus monkey identifies SLPI as a progesterone-regulated gene.[20]

We also report that elafin and SLPI mRNA expression levels are particularly high in the postpartum period following a normal full-term pregnancy. Over the course of gestation, the uterine cervix undergoes dramatic morphologic changes that allow the closed rigid structure that retains the fetus in the expanding uterus to soften and dilate for delivery. Although the molecular mechanisms that control the remarkable tissue changes involved in cervical remodeling are not fully understood, it is widely held that pro-inflammatory mediators are involved. Macrophages and neutrophils infiltrate the cervix prior to parturition. Their release of a variety of inflammatory signals induces the local production of a variety of proteases, including matrix metalloproteinase,[21] and granulocyte elastase[22] that sets in motion the local tissue remodeling known as cervical ripening.[23] After delivery, the cervix rapidly returns to its original rigid structure during the postpartum period. The antiprotease activities of the anti-inflammatory AMPs, elafin and SLPI, make them uniquely poised to participate in recovery.[9] The postpartum increase in elafin and SLPI transcription seen in this study may be important in the resolution of inflammation and the reconstruction of extracellular matrix involved in the resolution of the uterine cervical changes required for delivery.

We have also shown here that mRNA expression of elafin and SLPI in cervical cells is elevated in women whose pregnancies ended in preterm delivery but not in the women who delivered at term after in-hospital care for TPL. Although all samples were obtained from women when they presented with TPL, the changes in SLPI and elafin expression were seen both in women complaining of contractions and in women with short cervical length without uterine contractions, making it unlikely that the changes were merely a response to uterine contractions. In past studies of these WAP family protease inhibitors in chronic lung disease, inflammation was shown to enhance elafin and SLPI expression.[24] As ascending genital tract invasion by infectious microbes is a frequent finding in preterm birth, enhancements in elafin and SLPI expression in the uterine cervix may be a consequence of an

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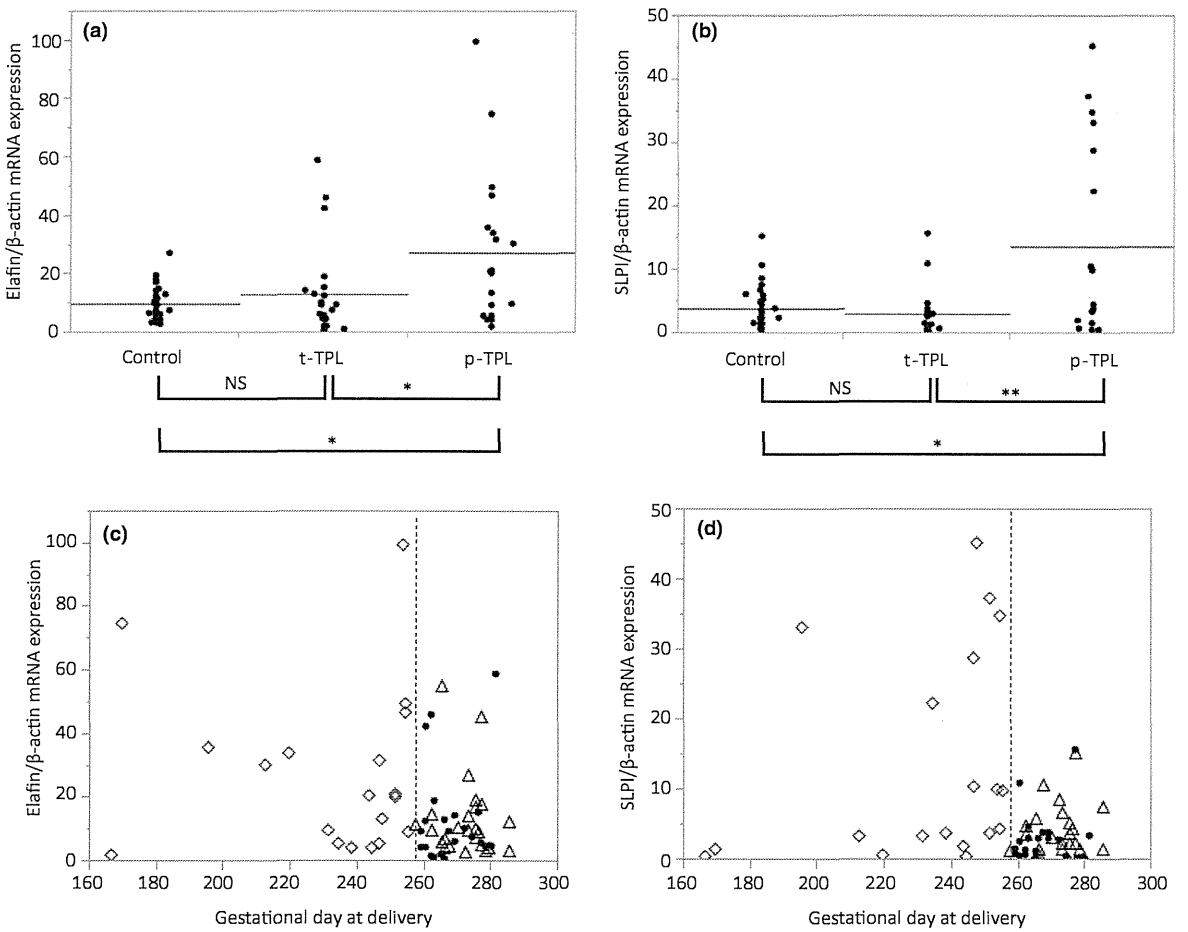

Fig. 3 Comparison of the mRNA expression of elafin and secretory leukocyte peptidase inhibitor (SLPI) in uterine cervical cells isolated from women with threatened preterm labor and normal pregnancies. Differences in cervical cell mRNA expression of elafin and SLPI in women with threatened preterm labor or normal pregnancies were assessed by real-time RT-PCR. Subjects with threatened preterm labor were separated into two groups based on their pregnancy outcome, t-TPL: term delivery (n = 23) and p-TPL: preterm delivery (n = 19). The bars in the charts depict mean mRNA levels in each group. Cervical cell samples from women with uncomplicated pregnancy were selected as controls (n = 26). (a) Elafin mRNA expression. (b) SLPI mRNA expression. (c) and (d) Association of elafin (c) and SLPI (d) mRNA expression levels with the gestational age at delivery is demonstrated on dot plots. Open squares: p-TPL, open triangles: t-TPL, filled circles: control. Dotted line in each plot indicates gestational age at day 259 (37 weeks and 0 day).

inflammatory tissue reaction to exogenous pathogens. Our results on elafin expression in the women with preterm labor agree with the study by Abbott et al.,[25] in which high elafin protein concentrations were detected in cervicovaginal fluid from women who developed a short cervix and had spontaneous preterm labor. Others have reported reduced SLPI and increased neutrophil elastase concentrations in the amniotic fluid of women with preterm rupture of membranes.[26,27] This SLPI protein change in amniotic fluid differs from our SLPI mRNA result in cervical cells. This discrepancy might be attributed to differences in sample types and methods of detection, because the expression analysis is performed only at mRNA level but not at protein level in this study. Another possible reason for discrepancy between our cervical cell mRNA and historical amniotic fluid protein results might be an underestimation of SLPI protein production due to local coexisting neutrophil elastase which is known to increase under the inflammatory environment of CAM preceding preterm labor.[22] Neutrophil elastase

has been shown to interfere with the secretion of SLPI *in vitro*, presumably by forming a positively charged molecular complex with SLPI.[28,29] The possibility for the interaction between neutrophil elastase and SLPI protein production remains to be clarified.

Although the women with preterm labor in our study displayed elevations in the cervical expression of elafin and SLPI mRNA, a given woman with high elafin expression did not necessarily also have high SLPI expression and vice versa. This suggests that the expression of these two AMPs may have distinct regulatory mechanisms. Supporting this hypothesis are the documented differences in the response patterns of elafin and SLPI to inflammatory signals in lung epithelial cells.[30] More mechanistically, the upregulation of elafin mRNA in response to inflammatory cytokines has been linked to the presence of a nuclear factor-κB site in its promoter region,[31] whereas the activation of signal transducers and activator of transcription 1 (STAT1) has been implicated in enhanced SLPI transcription in nasal epithelial cells from smokers.[32]

In conclusion, constitutive expression of elafin and SLPI in cervical cells during pregnancy suggest that they might play essential roles in local tissue homeostasis and immune defense. The elevations in cervical elafin and SLPI expression in the women who delivered at preterm might reflect the local response to the pathogen invasion into the cervix preceding preterm labor. Future study is necessary to clarify the functional roles of elafin and SLPI in mucosal immune defense at the uterine cervix during pregnancy.

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