Production of secretory leukocyte protease inhibitor by human amniotic membranes and regulation of its concentration in amniotic fluid

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Secretory leukocyte protease inhibitor (SLPI) is a potent inhibitor of human leukocyte elastase. SLPI is a protein found in various human fluids, including parotid secretions, cervical mucus, seminal plasma and ascites. Western blot analysis revealed that SLPI protein is detected as a 12 kDa band in both the amniotic fluid and the amniotic membrane. The amniotic fluid concentrations of SLPI were assayed by enzyme-linked immunosorbent assay. SLPI concentrations in the amniotic fluid of women in the third trimester were higher than those in the second trimester. Immunohistochemistry using an anti-SLPI polyclonal antibody revealed positive staining in epithelial cells in amniotic membranes. Reverse transcription-polymerase chain reaction demonstrated that SLPI transcripts could be detected in the amniotic membranes. To determine the mechanism of SLPI production by amniotic cells, purified amniotic cells were stimulated with various cytokines. Amniotic cells produced SLPI in a dose-dependent manner when stimulated with interleukin (IL)-1\(^\alpha\), IL-1\(^\beta\), and tumour necrosis factor-\(\alpha\). The present findings show that SLPI is produced by the amniotic membranes in response to cytokine concentrations. The SLPI in the amniotic fluid may contribute to immunodefence mechanisms during pregnancy.

Key words: amnion/amniotic fluid/amniotic membrane/secretory leukocyte protease inhibitor

Introduction

Secretory leukocyte protease inhibitor (SLPI), which has been found to be a potent inhibitor of human leukocyte elastase, human cathepsin G, and human trypsin (Thompson and Ohlsson, 1986), is a 12 kDa protein that was originally isolated from human parotid gland secretions (Thompson and Ohlsson, 1986). SLPI has also been shown to inhibit mast cell chymase (Fink \textit{et al}., 1986), a protease released during mast cell degranulation, and to inhibit histamine release from mast cells \textit{in vitro} (Dietze \textit{et al}., 1990). SLPI is a protein found in various fluids, including parotid secretions (Thompson and Ohlsson, 1986), cervical mucus (Helming \textit{et al}., 1995), seminal plasma (Ohlsson \textit{et al}., 1995; Moriyama \textit{et al}., 1998) and ascites (Shimoya \textit{et al}., 2000). The gene for SLPI is expressed in a tissue-specific manner by cells at a variety of mucosal surfaces such as those of the lung, cervix, parotid duct, and seminal vesicles (Abe \textit{et al}., 1991). The concentrations of SLPI in biological samples have been monitored to correlate these concentrations with pathological conditions (Kida \textit{et al}., 1992; Kouchi \textit{et al}., 1993; Sluis \textit{et al}., 1994). Increased concentrations of SLPI in nasal secretions and in bronchoalveolar lavage fluids may be indicative of inflammatory lung conditions or allergic reactions (Fryksmark \textit{et al}., 1989; Vogelmeier \textit{et al}., 1991; Lee \textit{et al}., 1993). We have also reported that the SLPI molecule modulates immunodefence function in the male and female genital tracts (Moriyama \textit{et al}., 1998, 1999). Ohlsson \textit{et al}., have suggested that SLPI has a local protective function against proteolytic degradation of the male reproductive tract tissues (Ohlsson \textit{et al}., 1995). Recently, a study using a knock-out mouse model demonstrated that this molecule is strongly associated with the wound-healing process (Ashcroft \textit{et al}., 2000).

Amniotic fluid contains various cytokines, such as interleukin (IL)-1, tumour necrosis factor (TNF)-\(\alpha\) and IL-6 (Baud \textit{et al}., 1999; Gonzalez-Bosquet \textit{et al}., 1999). In amniotic fluid, several offensive factors, including cytokines and proteases, mediate various conditions such as preterm labour, premature membrane rupture, and pre-eclampsia (Cox \textit{et al}., 1997; Arntzen \textit{et al}., 1998). The SLPI molecule is able to modulate immunodefence function in various organs (Ohlsson \textit{et al}., 1995; Moriyama \textit{et al}., 1998, 1999). We have also reported that various cytokines are produced by placental
cells in the inflammatory state (Shimoya et al., 1998, 1999). Although many studies have demonstrated the role of offensive factors including cytokines during pregnancy, there have been few studies on defensive factors, such as SLPI, during pregnancy. Recently, it has been reported that the concentration of SLPI in amniotic fluid increased significantly from the second trimester to the third trimester (Denison et al., 1999), and SLPI was shown to be released by decidua and chorion-decidua (Denison et al., 1999). The aim of this study is to investigate the production of SLPI by the amnion and to study the regulation of this production during pregnancy.

Materials and methods

Reagents

Goat anti-SLPI polyclonal antibodies and recombinant (r)SLPI were purchased from R&D systems (Minneapolis, MN, USA). Recombinant elastase was purchased from Sigma Chemical Co. (St Louis, MO, USA). A control goat IgG for the control of the immunohistochemical analysis was purchased from Zymed Laboratories (San Francisco, CA, USA).

Samples

Amniotic fluids were obtained from 15 pregnant women in the second trimester and from 12 pregnant women in the third trimester. Amniocentesis was performed for diagnosis of karyotype and fetal lung maturation. Nine samples of amniotic membranes were obtained from pregnant women at term who underwent elective Caesarean section without medical complications. This study was approved by the local ethics committee of the Department of Obstetrics and Gynecology, Faculty of Medicine, Osaka University. Informed consent was obtained from each patient. Amniotic fluids were centrifuged (700 g for 10 min) to separate the cell pellet and the supernatants were collected. The supernatant was then stored at −20°C until assayed.

Tissue preparation for Western blot analysis

The homogenizing buffer for protein extraction from the amniotic membranes consisted of 0.5 mol/l Tris–HCl (pH 6.8), 10% sodium dodecyl sulphate (SDS), β-mercaptoethanol, and 1% bromophenol blue (BPB). The amniotic membranes were homogenized in a 2 ml volume. Homogenates were centrifuged at 4°C for 30 min at 14 000 g to remove debris. Following protein determinations, the samples were aliquoted, and subjected to polyacrylamide gel electrophoresis (PAGE).

Western blot analysis of amniotic fluids and amniotic membranes

To determine SLPI protein in the amniotic fluids and the amniotic membranes, we performed a Western blotting analysis using an anti-human SLPI polyclonal antibody. Amniotic fluids (10 µl) and ~10 µg of amniotic membrane protein were electrophoresed on 15% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes (0.45 µm; Schleicher and Schuell, Dassel, Germany). The membrane was incubated with 5% dried milk protein followed by goat anti-human SLPI polyclonal antibody. The primary antibody was used at a final concentration of 1.0 µg/ml. A horseradish peroxidase-linked anti-goat IgG antibody was then used as the secondary antibody. The SLPI immunoreactivity was visualized using an enhanced chemiluminescence (ECL) Western blotting analysis system (Amersham, Aylesbury, UK).

Protein assay

Protein concentrations were determined with Bio-Rad (Hercules, CA, USA) Protein Determination Reagent, according to a published method (Bradford, 1976).

Determination of SLPI by enzyme-linked immunosorbent assay (ELISA)

To determine concentrations of SLPI in the amniotic fluids and the supernatants of amniotic cell culture, ELISA kits specific for SLPI (R&D Systems) were used. The concentrations of SLPI detected covered amounts of >25 pg/ml. No cross-reactivity with cytokines, such as IL-1, IL-6 and IL-8, chemokines, such as MCAF, growth factors, such as epidermal growth factor (EGF), or proteases, such as leukocyte elastase, trypsin and chymotrypsin could be found in this kit. Intra-assay variability with the SLPI kit was 4.2–8.0%, and the inter-assay variability was 4.9–8.0%.

Determination of elastase titre by ELISA

To measure titres of elastase in amniotic fluids, ELISA kits specific for elastase (Merck, Darmstadt, Germany) were used. Amniotic fluid titres of elastase detected by the kit covered the ranges of over 1.0 µg/l. Intra-assay variability with the elastase kit was 2.7–5.2%, and the inter-assay variability was 4.9–9.5%.

RNA extraction

RNA was extracted from amniotic membrane samples of 0.5 g wet weight by acid guanidinium thiocyanate-phenol-chloroform extraction according to a published method (Chomczynski and Sacchi, 1987).

RT-PCR amplification

RT-PCR was performed using an RT-PCR high kit (Toyobo Co., Tokyo, Japan). The reaction was carried out in the presence of M-MLV RTase and 1 ml RNA sample in a 5×RTase buffer, random primers, and dNTP mix for 40 min at 42°C. PCR amplification was performed, using an RT mixture (10 ml), with sequence-specific primers against human SLPI (5′-ACTCTCTCCTTCACACTGAA-3′/5′-CATTGGATCAA-CTGGCACTT-3′). PCR was carried out for 35 cycles using a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT, USA). Each cycle consisted of denaturation at 94°C (40 s), annealing at 52°C (40 s), and extension at 72°C (40 s). The amplification yielded a 570 bp DNA product according to the published sequence of the SLPI gene (Stetler et al., 1986). RT was performed with total RNA without reverse transcriptase (a mock RT sample) to detect possible contamination in RNA samples by genomic DNA. Twenty microlitres from each of the 50 µl PCR mixtures was electrophoresed on 1% agarose gel, then stained using ethidium bromide, and amplified products were visualized by UV illumination. PCR products were digested by BamHI to confirm that they were specific SLPI transcripts. Molecular sizes were estimated using a 100 bp DNA ladder. All primers were obtained from Becks (Tokyo, Japan).

Immunohistochemical staining of SLPI in the amniotic membranes

To determine the localization of SLPI in the amniotic membrane, we performed immunohistochemical staining using an avidin-biotin peroxidase complex method kit (Omini/Tags Universal Streptavidin/Biotin Affinity Immunostaining Systems, Lipshaw, Pittsburg, 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) or control goat IgG for the control was applied at room temperature and left for 1 h. After rinsing with phosphate-buffered saline (PBS), the sections were further incubated for 30 min with biotin-labelled goat anti-mouse IgG, followed by the addition of avidin-peroxidase complex at 4°C. Peroxidase activity in the sections was visualized with 0.1% 3,3-diaminobenzidine-tetrahydrochloride containing 0.02% hydrogen peroxide in 0.1 mol/l Tris buffer (pH 7.2). The slides were counterstained with Mayer’s haematoxylin.
Preparation of amniotic cells

To measure the level of SLPI protein production by amniotic cells, single cell suspensions were prepared as described previously (Bry et al., 1994; Hammer et al., 1997). Briefly, the amniotic membranes were peeled off chorion-decidua, minced into pieces and incubated in a solution containing 0.05% trypsin for 1 h at 37°C. The dissociated cells were filtered through gauze and washed three times with PBS. After resuspension in Roswell Park Memorial Institute 1640 medium with 10% fetal calf serum, 2 mmol/l glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, amniotic cells were cultured in 24-well culture plates at 37°C.

Stimulation of amniotic cells

The prepared amniotic cells were adjusted to 5×10^5/ml and immediately stimulated with various concentrations of IL-1α, IL-1β or TNF-α. The culture supernatants were collected after stimulation with cytokines for 12, 24 or 48 h, centrifuged and filtered. The cell-free culture supernatants were then frozen at −80°C until measurement of SLPI concentrations by ELISA. After treatment, the viability of the amniotic cells, as detected by Trypan Blue dye exclusion, was >80%.

Statistical analysis

Statistical analyses were conducted using Student’s t-test, Duncan’s new multiple range test or Welch’s t-test, and P < 0.05 was considered significant. Correlation was analysed by simple linear regression. The values given represent the means ± SEM.

Results

To quantify SLPI protein in the amniotic fluids, we performed Western blot analysis. As shown in Figure 1, SLPI protein was detected as a 12 kDa band in the amniotic fluids. A second smaller immunoreactive band was also detected by Western blot analysis. This band is thought to represent a degradation product of SLPI as it was detected in the amniotic fluid but not in the amniotic membranes (see below). These findings show the presence of SLPI in the amniotic fluids. Table I shows the SLPI titres in the amniotic fluids of pregnant women during the second (n = 15) and the third trimesters (n = 12). The SLPI titres in the amniotic fluids of pregnant women in the third trimester (802 ± 138 ng/ml) were significantly higher than those in the second trimester (106 ± 15 ng/ml) (P < 0.0001). To examine the correlation between SLPI and elastase concentrations in amniotic fluids, a simple linear analysis was performed. As shown in Figure 2, there was no correlation between SLPI and elastase concentrations in the amniotic fluids of pregnant women. The ratio of SLPI/elastase in the amniotic fluid during the third trimester (0.52 ± 0.12) was also significantly higher than that during the second trimester (0.073 ± 0.013) (P < 0.001) (Table I).

As shown in Figure 3, Western blot analysis also detected SLPI protein in the amniotic membranes as a 12 kDa band. There was no significant difference between the intensity of SLPI in the amniotic membranes during the second trimester (n = 4) and the third trimesters (n = 6). RT-PCR was performed to determine the expression of the SLPI gene in the amniotic membranes during pregnancy. Figure 4 shows that SLPI transcripts were present in the amniotic membranes. The 570 bp PCR products were digested by BamHI to produce fragments of 336 and 234 bp, thus confirming that they were exact SLPI gene transcripts (data not shown). To identify the origin of this large amount of SLPI, we performed immunohistochemical staining of sections of the amniotic membranes in the third trimester,
Figure 4. Secretory leukocyte protease inhibitor (SLPI) mRNA expression in amniotic membranes during pregnancy. Agarose gel electrophoresis of polymerase chain reaction-amplified DNA of SLPI. Lane 1, DNA size marker: 100 bp ladder. cDNA was prepared from the amniotic membranes from women at 35 gestational weeks (lane 2), at 36 gestational weeks (lane 4), and at 38 gestational weeks (lane 6). Mock reverse transcription-polymerase chain reaction was also performed with cDNA samples from the amniotic membranes from women at 35 gestational weeks (lane 3), at 36 gestational weeks (lane 5) and at 38 gestational weeks (lane 7). Lane 8, DNA size marker: 100 bp ladder.

using an anti-SLPI polyclonal antibody. The epithelial cells in the amniotic membranes were intensely stained (Figure 5).

To investigate the regulation of SLPI production in the amniotic fluids, amniotic cells were cultured with various concentrations of IL-1α, IL-1β and TNF-α. Figure 6A demonstrates the kinetics of SLPI production by amniotic cells stimulated with IL-1α. Amniotic cells produced a significantly increased amount of SLPI after 24 h of stimulation with IL-1α (P < 0.05 versus control). An even larger amount of SLPI production after 48 h of stimulation with IL-1α was observed (P < 0.05). Figure 6B demonstrates the dose-dependent effect of IL-1α on SLPI production by amniotic cells. Figure 7A shows the kinetics of SLPI production of amniotic cells stimulated with IL-1β. Amniotic cells produced a significantly increased amount of SLPI after 48 h of stimulation with IL-1β (P < 0.05 versus control). As shown in Figure 7B, amniotic cells stimulated with IL-1β produced SLPI in a dose-dependent fashion. Figure 8A demonstrates the kinetics of SLPI production of amniotic cells stimulated with TNF-α. Amniotic cells produced a significantly increased amount of SLPI after 24 h of stimulation with TNF-α (P < 0.05 versus control). An even larger amount of SLPI production after 48 h of stimulation with TNF-α was observed (P < 0.01 versus control). Figure 8B shows that TNF-α induced SLPI production by amniotic cells in a dose-dependent fashion.

Discussion
In the present study, a Western blot analysis revealed that SLPI protein in the amniotic fluid during the second and the third trimesters was expressed. A certain amount of SLPI is contained in human amniotic fluid, and this is consistent with the observations of a previous study (Denison et al., 1999). Also in agreement with Denison et al., the SLPI titre of the amniotic fluid during the third trimester was found to be significantly higher than that during the second trimester. These findings suggest that the SLPI molecule is constitutively present in the amniotic fluid.

Proteases and protease inhibitors play essential interactive roles during the inflammatory process. SLPI, which is a potent inhibitor of human leukocyte elastase, human cathepsin G, and human trypsin (Thompson and Ohlsson, 1986), participates in the body’s natural defence. This molecule might contribute to homeostasis in the amniotic fluid.

Neutrophil elastase has been shown to increase SLPI transcript concentrations in primary human airway epithelial cells, whereas TNF-α and IL-8 produced little or no effect on SLPI transcript concentrations (Abbinante et al., 1993). Elastase is a strong protease which is produced by leukocytes in amniotic fluid (Matsuda et al., 1995; Akutsu and Iwama, 2000). Recently, it was demonstrated that elastase concentrations in amniotic fluid are a reliable index to estimate the occurrence of rupture of membranes (Akutsu and Iwama, 2000). The relationship between elastase in amniotic fluid and perinatal infections has been reported (Matsuda et al., 1995). However, no information about the relationship between SLPI and elastase in the amniotic fluid was available. In the present study, we could not find any correlation between elastase and SLPI. Amniotic fluid has
bacteriostatic and bacteriocidal properties. The SLPI/elastase ratio in the amniotic fluid in the third trimester was significantly higher than that in the second trimester. The up-regulation of SLPI has been shown to play a defensive role in the epithelial surface of inflammatory lung diseases (Abbinante et al., 1993). Thus, SLPI might protect the amniotic epithelium from the...
leukocyte protease of amniotic fluid when uterine contractions or intrauterine infections occur. We previously reported the beneficial effect of SLPI in the cervical mucus during the menstrual cycle and that SLPI might constitute the major defence of cervical tissues (Moriyama et al., 1999). It may be more appropriate to see it as having a protective role in preventing the development of uterine contractions and rupture of membranes through various mechanisms such as macrophage digestion and the release of inhibitory factors. Further investigations will be necessary to examine the relationship between SLPI concentrations and uterine concentration/rupture of membranes.

Amniotic fluid has an important role in fetal lung function and maturation. Elevated amniotic fluid TNF-α concentrations have been associated with respiratory distress syndrome (RDS) and prenatal exposure to TNF-α is a risk factor for RDS (Hitti et al., 1997). It has been reported that antenatal exposure to proinflammatory cytokines is a risk for the development of bronchopulmonary dysplasia (Yoon et al., 1997). The SLPI molecule plays a defensive role in the epithelial surface of inflammatory lung diseases (Abbinante et al., 1993). The SLPI molecule in amniotic fluid might play a defensive role during the development of fetal lung function in addition to a defensive role to prevent premature rupture of membranes. Further investigations are necessary to determine the SLPI function on fetal lung.

To determine the source of SLPI in amniotic fluid, we performed Western blotting and immunohistochemical analysis using an anti-human SLPI polyclonal antibody. The Western blot analysis detected SLPI protein in the amniotic membranes as a 12 kDa band. We also found that the SLPI gene was expressed in the amniotic membranes. However, we did not detect a significant difference in SLPI gene expression during pregnancy by RT-PCR. The SLPI gene 5′ flanking region has a TATA box and multiple potential AP-1 binding sites which are capable of mediating a specific response to the induction by phorbol esters. However, the mechanisms controlling SLPI gene expression in vivo are unknown (Abe et al., 1991). The immunohistochemical analysis showed that the epithelial cells of the amniotic membrane were intensely stained, indicating that these epithelial cells were the main source of SLPI in the amniotic fluid. The present findings of the immunohistochemical analysis are consistent with previous observations (Helming et al., 1995; Denison et al., 1999).

Various studies have demonstrated that the amniotic fluid of pregnant women contains high amounts of cytokines (Keelan et al., 1997; Arntzen et al., 1998; Baud et al., 1999; Gonzalez-Bosquet et al., 1999). It has been suggested that IL-1 concentrations in the amniotic fluid are increased with labour (Cox et al., 1997; Allport et al., 2000). In mice, SLPI transcript and protein were detected in peritoneal macrophages, bone marrow-derived macrophage cell lines and peritoneal polymorphonuclearocytes (Jin et al., 1997) and an anti-inflammatory role for macrophage-derived SLPI appears likely based on the slow production of SLPI in response to constituents of Gram-negative and Gram-positive bacteria (Jin et al., 1997). Several inflammatory mediators can enhance SLPI mRNA concentrations and the production of SLPI protein in HS-24 human bronchial epithelial cell line (Maruyama et al., 1994). We previously reported that placental cells produced various cytokines in placental infection (Shimoya et al., 1998, 1999). In the present study, amniotic cells produced SLPI in response to IL-1α, IL-1β and TNF-α. These findings demonstrate that the SLPI molecule in the amniotic fluid is regulated by cytokines in the amniotic fluid. This might be one reason why the SLPI concentrations in amniotic fluids during the third trimester are higher than those during the second trimester. However, it is still unknown as to whether cytokine treatment induced the synthesis of SLPI de novo or just increased secretion of the pre-formed protein. Further investigations of SLPI gene expression by cytokine-stimulated cell culture systems will be necessary to determine this point.

In summary, the SLPI molecule is produced by amnion in response to increased cytokine in amniotic fluids. This molecule may play an important role during pregnancy to protect from protease and inflammatory cytokines which could lead to premature rupture of membranes and uterine contractions. In addition to these functions, the SLPI molecule might play a defensive role during the development of fetal lung function.

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References


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