Human cervical ripening, an inflammatory process mediated by cytokines

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An extensive remodelling process, referred to as cervical ripening, takes place in the cervical tissue during pregnancy and labour. It is recognized as softening and dilation of the cervical canal, and starts as a slow process during pregnancy, becoming rapid close to partum. In this study we focus on cytokines as possible mediators of this final remodelling. mRNA levels for interleukin (IL)-8, IL-6 and granulocyte colony-stimulating factor (G-CSF) were upregulated in the ripe postpartum cervical tissue (n = 8) compared to the unripe state (n = 9). Likewise, released cytokine concentrations increased from non-pregnant (n = 11) to the term-pregnant group (n = 13) with a further increase at partum (n = 16). IL-8 concentrations increased 4-fold from non-pregnant to term-pregnant (P < 0.01), and a further 10-fold to postpartum state (P < 0.0001). Concentrations of IL-6 and G-CSF were similarly increased. Specific IL-8 immunostaining was identified in the epithelia of pregnant cervical tissue (n = 7) and was most pronounced in the epithelia and stroma of postpartum tissue (n = 4). In conclusion, IL-8, IL-6 and G-CSF increase in the human cervix during the ripening process, indicating their important role in the cervical remodelling. These data demonstrate that cervical ripening is similar to an inflammatory process.

Key words: cervical ripening/cytokines/IL-8/IL-6/G-CSF

Introduction

A prerequisite for a normal onset and progress of labour is sufficient cervical ripening in coordination with uterine contractions. Disturbances in this process cause major clinical problems, such as dysfunctional and protracted labour due to insufficient cervical softening (Ekman et al., 1986). Another obstetric problem is premature delivery, characterized by premature cervical ripening followed by an early onset of labour.

The human cervix, in contrast to the uterus, is essentially a fibrous connective tissue organ, mainly composed of collagen and proteoglycans. The connective tissue content is ~90–95% in the lower part of the human cervix and ~75% in the isthmic region of the uterus (Granström et al., 1989). Cervical ripening occurs in two steps: a slow stage extending over the major part of the pregnancy, and a final rapid process just preceding labour. The slow process involves a change in turnover of matrix components and results in a reorganization (Uldbjerg et al., 1983; Granström et al., 1989) of the collagen fibrillar network. During cervical ripening there is a 50–70% decrease in collagen and proteoglycan concentrations concomitantly with an increase in collagen synthesis. This higher proteolytic activity coincides with an increase in the solubility of collagen (Uldbjerg et al., 1983; Granström et al., 1989). The final ripening is characterized by an influx of neutrophils (Junqueira et al., 1980) capable of secreting collagenase and elastase. This final remodelling is necessary for a harmless and successful delivery of the fetus.

The mediators of the cervical ripening process are still largely unknown, but prostaglandin (PG) E2 (Calder and Embrey, 1973; Ulmsten et al., 1982) and hormones such as oestrogen (Stjernholm et al., 1996) enhance ripening whereas progesterone (Rådestad et al., 1990; Sato et al., 1991) is a negative factor. Increased IL-8 concentrations were found in isthmus uteri in pregnant women during labour and cervical dilation (Winkler et al., 1998). These results are interesting and in congruence with the uterine connective tissue remodelling shown earlier (Granström et al., 1989). The ripening process of the uterine connective tissue is also crucial for normal labour (Granström et al., 1991). Several studies implicating inducible NO synthetase to be an important mediator in the cervical ripening process (Calder, 1998; Chwalisz and Garfield, 1998; Norman et al., 1998; Romero, 1998) also support the hypothesis that inflammatory mediators are important factors for the final cervical remodelling.

Here we investigate whether the final cervical ripening is an inflammatory process mediated by cytokines such as interleukin (IL)-8, IL-6 and granulocyte colony-stimulating factor (G-CSF). In a previous study, we showed an 11-fold increase of IL-8 protein in ripened, compared to unripened...
cervix (Sennström et al., 1997). In this study we examine upregulation of gene expression as well as protein concentrations of IL-8, IL-6 and G-CSF in ripe cervical tissue.

Materials and methods

Patients

Fifty-five healthy women were, after informed consent, included in the study. The Ethics Committee at the Karolinska Hospital approved the study.

Sixteen non-pregnant fertile women, regularly menstruating, with a median age of 44 years (range 39–50), and a median parity of 1 (range 0–3) admitted for hysterectomy for non-malignant diseases, were designated as the non-pregnant group. The term-pregnant group consisted of 19 term-pregnant women undergoing elective Caesarean section due to fetopelvic disproportion, breach presentation or earlier Caesarean section. All had unripe cervices, intact membranes and no sign of labour. All were nulliparous, except one who had had an earlier vaginal delivery. The median age was 32 years (range 26–42) and the median age in gestation was 270 days (range 261–282). Cervical score (a modified Bishop score), was low with median 2.0 points (range 0–6.0). The median fetal weight was 3470 g (range 3060–4325). In the group referred to as the postpartum group, 20 term-pregnant women with a spontaneous cervical ripening and a spontaneous onset of labour were included. All women had a normally proceeding labour, with a normal vaginal delivery, except for four women where vacuum extraction was used due to threatening fetal hypoxia. Median age was 31 years (range 24–38) and median parity 0 (range 0–3). The median gestational age was 282 days (range 267–297) and the time from regular contractions until parturition was median 9.5 h (range 2.0–17.0). During labour, 11 of the women had epidural anaesthesia. Gestational age was determined with ultrasonographic screening in the 17th–19th gestational week in all the pregnant women.

Tissue samples

Cervical biopsies weighing ~300 mg were taken from the anterior lip of the cervix by scissors and tweezers. The biopsies were taken transvaginally immediately after Caesarean section and within 15 min after vaginal parturum. Cervical biopsies from the non-pregnant women were taken just after removal of the uterus during hysterectomy. Cervical biopsies from the non-pregnant women were designated as the non-pregnant group. The term-pregnant group consisted of 19 term-pregnant women undergoing elective Caesarean section due to fetopelvic disproportion, breach presentation or earlier Caesarean section. All had unripe cervices, intact membranes and no sign of labour. All were nulliparous, except one who had had an earlier vaginal delivery. The median age was 32 years (range 26–42) and the median age in gestation was 270 days (range 261–282). Cervical score (a modified Bishop score), was low with median 2.0 points (range 0–6.0). The median fetal weight was 3470 g (range 3060–4325). In the group referred to as the postpartum group, 20 term-pregnant women with a spontaneous cervical ripening and a spontaneous onset of labour were included. All women had a normally proceeding labour, with a normal vaginal delivery, except for four women where vacuum extraction was used due to threatening fetal hypoxia. Median age was 31 years (range 24–38) and median parity 0 (range 0–3). The median gestational age was 282 days (range 267–297) and the time from regular contractions until parturition was median 9.5 h (range 2.0–17.0). During labour, 11 of the women had epidural anaesthesia. Gestational age was determined with ultrasonographic screening in the 17th–19th gestational week in all the pregnant women.

Measurement and quantification of mRNA for IL-8, IL-6 and G-CSF

Northern blot analysis

The tissue was homogenized on ice in guanidinium isothiocyanate for 30 s at 20 000 rpm. with a Buhler knife homogenizer (Polythron®). Total RNA was isolated using a published method (Chomczynski and Sacchi, 1987). Equal amounts of RNA (20–30 µg/mel) were separated by electrophoresis and transferred to nylon filters according to standard procedures (Sambrook et al., 1989). After electrophoresis, part of a gel containing one series of samples (two non-pregnant, two term-pregnant and two postpartum) was stained with ethidium bromide to verify that equal amounts of RNA were applied in each well, and that the RNA was intact. After destaining it was processed as described below. This treatment did not affect the level of hybridization (data not shown). The filters were further hybridized with specific 32P-labelled cDNA probes overnight at 42°C (Westergren-Thorsson et al., 1991; Tiedemann et al., 1997). The 32P-labelled probes had a specific activity of ~8x10^5 c.p.m./µg DNA and were prepared by end labelling, using T4 polynucleotide kinase (Gibco, BRL, Täby, Sweden). Oligonucleotide DNA probes of 30 bases of human IL-6, IL-8 and G-CSF were purchased from R & D Systems, Abingdon, UK. The filters were washed sequentially with 2×SSC (0.15 mol/l sodium chloride, 15 mmol/l sodium citrate, pH 7.0), –0.05% sodium dodecyl sulphate at room temperature, and with 0.2×SSC, –0.1% sodium dodecyl sulphate at 50–56°C. The radioactivity was visualized and intensity was measured by a Fuji BAS 2000 bioimage plate analyser. The radioactivity was further related to the sum of the intensity of the 18S and 28S rRNA bands from the ethidium bromide gel by scanning with gel-Pro™ Analyser version 2.0 (deLeeuw et al., 1989; Sahlin et al., 1994). The mRNA differences were presented as percentage of non-pregnant controls. On each gel two non-pregnant patients served as control. All the Northern blots could not be run at the same time, so the levels are semiquantitative and must be related to the control patients on the same gel.

Measurement of IL-8, IL-6 and G-CSF in the supernatant of homogenized cervical tissue

Preparation of homogenized cervical samples

The biopsies were weighed and cut into small pieces on dry ice. Phosphate-buffered saline (PBS) containing ~0.01 ng/ml endotoxin when tested with Limulus amoebocyte lysate endochromes endotoxin method (Endosafe Inc., Charleston, SC, USA) was added at a ratio of 1000 mg cervix/ml PBS. Each sample was homogenized in the cold using Polytron PT 3000 for 15 s at 4000 r.p.m. or until complete homogenization was achieved. The homogenate was centrifuged at 300 g for 10 min at 4°C and the supernatant was stored at –70°C until assayed. As a control the pellet was resuspended in 0.5 mol/l NaCl to release IL-8 bound to heparan sulphate. The sample was then centrifuged and the assay performed.

Cytokine determination

Cytokines were determined by enzyme immunoassay (EIA). IL-8, IL-6 and G-CSF kits were obtained from R & D systems (Abingdon, Oxon, UK). The limit of detection for IL-8 was 31.2 pg/ml, for IL-6 3.12 pg/ml and for G-CSF 39 pg/ml. The median intra-assay variation for IL-8 was 4.6%, for IL-6 it was 3.1%, and for G-CSF 2.1%. The inter-assay variation was median 6.8% for IL-8, 2.5% for IL-6 and median 8.8% for G-CSF.

Immunohistochemical localization of IL-8 staining in cervical tissue

Cervical tissue from four non-pregnant, three term-pregnant and four postpartum patients were embedded in OCT compound (Tissue-Tek; Miles, Elkhart, IN, USA). Frozen sections, 8 µm thick, were mounted on gelchromatin-coated glass slides. Staining was performed as previously described by (Sanders et al., 1991). The sections were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, air-dried, and kept at –20°C until used. The slides were rehydrated in balanced salt solution (BSS; Gibco Ltd, Paisley, UK) containing Ca2+ and Mg2+ supplemented with 0.01 mol/l HEPES buffer. The endogenous peroxidase activity was blocked with 1% H2O2 and 3 mol/l NaN3 in BSS containing 0.1% saponin (Sigma Chemical Co., St Louis, MO, USA) as detergent, pH 7.4 (Sanders et al., 1991). Thereafter, the slides were incubated with 5 µg/ml of the monoclonal IL-8 antibody, NAP-1 (mouse immunoglobulin G1; Miroslav Ceska, Vienna, Austria) overnight in a humidified chamber at room temperature. Non-specific binding was eliminated by pre-blocking with 1% normal goat serum (Sigma). Biotinylated goat anti-mouse immunoglobulin G1 (IgG1) (Caltag Laboratory, South San Francisco, CA, USA), diluted 1:600, was used as secondary antibody. After further washing, the sections were treated with an

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Figure 1. Detection and quantification of cytokine mRNA in cervical tissue. (a) A representative Northern blot of the transcripts of interleukin (IL)-8 (1.8 kb), IL-6 (1.3 kb) and granulocyte colony-stimulating factor (G-CSF) (1.5 kb) from cervix are shown. Ethidium bromide (EtBr) staining of 18S and 28S rRNA are included to demonstrate that the same amount of RNA was applied in respective lanes. Two different non-pregnant patients were used as references on each gel, to prevent the risk of false positive results. NP, non-pregnant; TP, term-pregnant; and PP, postpartum cervical tissue. (b) Estimation of mRNA intensities after Northern blot analysis of cervices at different stages of ripening. mRNA analysis was performed on homogenized cervical tissue from eight non-pregnant women which served as controls, nine term-pregnant women (TP) and eight postpartum women (PP). mRNA differences of IL-8 (filled dots), IL-6 (unfilled dots) and G-CSF (unfilled squares) are expressed as percentage of non-pregnant controls related to the sum of rRNA 18S and rRNA 28S estimated by scanning of ethidium bromide filters. Each symbol represents a value from one patient. Median values are indicated (short horizontal lines). *P < 0.05, **P < 0.01, ***P < 0.001.

avidin–biotin–horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) and overlaid with 3,3’-diaminobenzidine (Vector Laboratories) as substrate chromagen and then incubated. The reaction field was blocked with BSS without saponin. Slides were counterstained with Mayer’s haematoxylin, mounted in a glycerine buffer and visualized by light microscopy. To test for staining specificity, highly purified recombinant IL-8 was used to block specific cytokine staining. The primary IL-8 antibody, 5 µg/ml, was incubated with recombinant IL-8 in 10-fold excess (50 µg/ml) at 4°C overnight. Staining was performed as previously described. Cytokine-producing areas were identified and photographed in a Polyvar 2 microscope (Reichert-Jung, Vienna, Austria). The entire tissue section was examined.

Statistical methods
The Mann–Whitney U-test was used to evaluate differences between groups. P < 0.05 was considered significant. Results are expressed as median values.

Results
Increased levels of mRNA for IL-8, IL-6 and G-CSF in postpartum cervical tissue
Northern blot analysis of IL-8, IL-6 and G-CSF showed clearly that mRNA was greatly increased in postpartum cervix compared to the mRNA levels in the non-pregnant control group (Figure 1a). However, in term-pregnant cervical tissue only small increases were noted (Figure 1b). By estimating intensities it could be shown that the mRNA levels of IL-8 increased >4-fold, from the non-pregnant to the postpartum state (P < 0.001; Figure 1b). The mRNA expression of IL-6 increased 7-fold (P < 0.001) and G-CSF increased >10-fold in the tissue postpartum, compared to the non-pregnant controls (P < 0.001; Figure 1b). A significant increase was also seen for IL-8, IL-6 and G-CSF when comparing the term-pregnant with the postpartum group. IL-8 increased 5-fold from term-pregnant to postpartum tissue (P < 0.01; Figure 1b), and IL-6 and G-CSF were upregulated >10-fold (P < 0.001; Figure 1b).

Increased IL-8, IL-6 and G-CSF concentrations in postpartum cervical tissue
In the supernatant of homogenized human cervical tissue IL-8, IL-6 and G-CSF concentrations were measured by enzyme-linked immunoassay. IL-8 increased >3-fold, from 680 pg/mg wet weight in the non-pregnant to 2400 pg/mg wet weight in the term-pregnant tissue (P < 0.01; Figure 2). In postpartum cervical tissue the concentration of IL-8 increased...
showed areas with abundant IL-8 staining throughout the whole section. In four out of four patients IL-8 was detected in the squamous epithelial cells (Figure 3c), in endothelial cells of venules and in stromal cells (Figure 3d). The specificity of the immune reaction was confirmed by preincubation of IL-8-specific monoclonal antibody with recombinant human IL-8 showing an extinction of the IL-8 signal, proving specific IL-8 staining in pregnant cervical tissue.

**Discussion**

The present study strongly supports our contention that the local ripening process in the human cervix is an inflammatory reaction. Our data clearly demonstrate that IL-8, IL-6 and G-CSF are upregulated in the human cervical connective tissue at term. The pronounced increase of mRNA levels in the postpartum fully ripened cervix supports an upregulation at the transcriptional level. We therefore suggest that cytokines play an active role in the connective tissue remodelling during the ripening process. Earlier investigations (Granström et al., 1989) have shown that the final connective tissue remodelling is accomplished before or during early labour (cervical dilation 3–4 cm) (Granström et al., 1989). The mechanisms involved in this process, clinically recognized as cervical softening and dilation, are still unclear. The ripening is related to a decrease in collagen and proteoglycan concentrations, and a several-fold increase in the collagen and proteoglycan turnover (Uldbjerg et al., 1983; Norman et al., 1993). During the final softening near the onset of labour, there is a more pronounced degradation, with an increased activity of proteolytic enzymes such as collagenase, stromelysin and serine elastase (Nagase et al., 1991). Fibroblasts as well as the relatively small amount of smooth muscle cells (15%) (Watari et al., 1999) may be one possible source for these enzymes, but they may also originate from polymorphonuclear granulocytes and macrophages. Our hypothesis is that cytokines are important in the modulation of the ripening process, and may be active in the connective tissue remodelling, which is supported by results from studies in other species (Junqueira et al., 1980; Chwalisz et al., 1994; El Maradny et al., 1996). Earlier studies demonstrating an increase of inflammatory cells, including granulocytes, in human cervix during the ripening process, further supports this theory (Liggins et al., 1981; Osmers et al., 1992; Knudsen et al., 1997).

**Abundant IL-8 expression in postpartum cervical tissue**

IL-8 was not detected in non-pregnant sections by immunohistochemical staining for IL-8 (Figure 3a). In the term-pregnant sections IL-8 staining was identified in the epithelium (Figure 3b). The most pronounced IL-8 staining was demonstrated in the postpartum tissue (Figure 3c,d). The staining additionally more than ten-fold to 26 200 pg/mg wet weight, compared with term-pregnant (P < 0.001; Figure 2). Concentrations of IL-6 increased >10-fold from the non-pregnant to term-pregnant states (73–843 pg/mg wet weight) (P < 0.001). In the postpartum state a further 15-fold increase to 15 100 pg/mg wet weight occurred (P < 0.0001; Figure 2). A >100-fold increase of IL-6 was noted between non-pregnant and postpartum biopsies (Figure 2). G-CSF concentrations increased from 1170 pg/mg wet weight in the non-pregnant to 4580 pg/mg wet weight in the term-pregnant state (P < 0.01), and there was a further 30-fold increase to 47 100 pg/mg wet weight in postpartum compared to non-pregnant cervical tissue (P < 0.0001; Figure 2). To release IL-8 bound to heparan sulphate, 0.5 mol/l NaCl was added to the homogenized pellets, and the supernatant was analysed for concentrations of IL-8. Cytokine concentrations varied in the same way between non-pregnant, term-pregnant and cervical tissue taken postpartum as in the former analysis.
epithelial cells, cervical tissue (Barclay et al., 1993) and chorio-decidual cells (Kelly et al., 1992; Dudley et al., 1993). An elevation of cytokines in different reproductive tissues during pregnancy has been shown (Romero et al., 1989; Ito et al., 1994; Saito et al., 1994; Arici et al., 1996; Axermo et al., 1996; Fortunato et al., 1996). Degradative enzymes such as serine proteases and metalloproteinases (MMP) are known to be released by IL-8, through degranulation of neutrophil granulocytes (Peveri et al., 1988; Nagase et al., 1991; Osmers et al., 1995; Zhang et al., 1998) in a process where IL-6 and G-CSF also are important. An earlier study (Winkler et al., 1999) showing an increase in the concentration of IL-8, MMP-8 and MMP-9 in the lower uterine segment during cervical dilatation further implicates an interaction of cytokines and MMP. In-vitro studies of human pregnant cervical fibroblasts also support this hypothesis (Ledingham et al., 1999).

In the present study we found an upregulation of proinflammatory cytokines. Possible mediators of this upregulation may be different hormones. During pregnancy, progesterone and oestrogen (Rådestad et al., 1990; Sato et al., 1991; Stjernholm et al., 1996) as well as PGE2 (Calder et al., 1973; Junqueira et al., 1980) and PGF2α are produced in the placental tissue. For many years, local administration of PGE2 was successfully used to induce cervical ripening in humans (Ekman et al., 1983), supporting a major impact of PGE2 upon this process.

In an extensive remodelling process such as cervical ripening, a potential role of proteoglycans must not be neglected (Norman et al., 1993). These macromolecules are known to regulate many different cell interactions such as cell migration, cell adhesion and signal transduction in the extracellular matrix (Winkler et al., 1999). Heparan sulphate proteoglycan, i.e. syndecan glypicans and perlecan, carries multiple binding sites for cytokines like IL-8 (Lindahl et al., 1994; Spillmann et al., 1998) and may therefore contribute to a local accumulation of the cytokine as well as protecting it from rapid degradation (Tanaka et al., 1993; Cadène et al., 1995; Clark et al., 1995). In concert with this, an interaction between cytokines and proteoglycans may be important for the regulation of cervical connective tissue reconstruction at term.

In conclusion, our results show an upregulation of the gene expression as well as the protein concentrations of IL-8, IL-6 and G-CSF in cervical tissue from pregnant women supporting the final ripening process to be an
inflammatory reaction. An interaction between inflammatory mediators and proteoglycans may also be of importance. Further studies are needed to investigate the pathways of this process, but we have shown that cytokines are important.

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