Quantitation and Localization of Inflammatory Cytokines Interleukin-6 and Interleukin-8 in the Lower Uterine Segment During Cervical Dilatation

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Purpose: Cervical dilatation during parturition is associated with a significant increase of interleukin (IL)-6 and IL-8. The purpose of this study was to localize the respective messenger RNA and to quantitate their protein levels during various stages of dilatation.

Methods: Protein extracts from 34 biopsy specimens of the lower uterine segment at various stages of cervical dilatation (<2, 2 to <4, 4–6, and >6 cm) were subjected to enzyme linked immunosorbent assay for IL-6 and IL-8 to document their concentration. The respective mRNA expression was studied using reverse transcriptase PCR using IL-6 and IL-8 specific primers. Localization of IL-6 and IL-8 was done by in situ hybridization using biotinylated probes.

Results: The IL-6 and IL-8 concentrations increased significantly in the lower uterine segment tissues at 4–6 cm of dilatation. Expression of IL-8 was seen in all the tissues at or more than 4 cm dilatation, whereas IL-6 expression was inconsistent. In situ hybridization localized IL-6 and IL-8 mRNA to the glandular epithelium.

Conclusions: The increase in the inflammatory cytokines (IL-6 and IL-8) during parturition is associated with cervical effacement.

KEY WORDS: Cervix; cytokines; IL-6; IL-8; messenger RNA; parturition.

INTRODUCTION

Cervical dilatation during parturition resembles an inflammatory reaction. It is characterized by an increasing number of granulocytes invading the cervical stroma as dilatation progresses. Granulocyte invasion is associated with the release of matrix degrading enzymes like collagenases and other proteases from their granules. These cells and the enzymes of their granules probably mediate the remodeling processes

within the extracellular matrix of the cervical connective tissue, which is the prerequisite for dilatation.

In this context the chemotactic cytokine interleukin (IL)-8 plays an important role. IL-8 induces granulocyte attraction, activation, and leukocyte degranulation. The subsequent release of various proteinases from their granules namely matrix metalloproteinase-8 (neutrophil collagenase) and matrix metalloproteinase-9 can degrade extracellular matrix components leading to cervical ripening (1). IL-8 can also be produced by human cervical tissue (2,3). Animal model studies have demonstrated IL-8 induced cervical ripening confirming its role in active labor (4).

IL-6, another inflammatory cytokine, is considered a marker for infection in the amniotic fluid (AF) even in the absence of clinical signs of infection (5).

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It is a product of decidua and amniochorion in response to intraamniotic infection (6). IL-6 provokes prostaglandin production in human amnion and decidua (7). However, a significant increase in the IL-6 concentration in AF has also been found after the onset of labor even in the absence of chorioamnionitis (8). The exact role of IL-6 and IL-8 in the initiation of either preterm or term labor and their role in cervical ripening are still unknown.

The purpose of this study was to determine the presence of IL-6 and IL-8 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR), to localize the mRNA by *in situ* hybridization, and to compare the results to the respective protein concentrations in the lower uterine segment during cervical dilatation.

METHODS

The study was approved by the ethics committee of the University Hospital of the Technical University of Aachen.

Specimen Collection

Biopsy specimens were taken from the lower uterine segment, which is representative of cervical tissue (9), of 34 women undergoing cesareans because of abnormal fetal heart rate patterns or cephalopelvic disproportion at term, 38–40 weeks gestation. The cervical tissues collected include dilatation <2 cm ($n = 9$), 2 to <4 cm ($n = 7$), 4–6 cm ($n = 8$), and >6 cm ($n = 10$). Cervical dilatation was assessed by palpation immediately before surgery by an experienced obstetrician between contractions. Patients with clinical or histological evidence of chorioamnionitis (fever of more than 38°C, elevation of leukocytes and/or C-reactive protein in AF, fetal tachycardia, untreatable contractions, positive microbiology (culture positive for microorganisms in the AF), or signs of chorioamnionitis in placenta histology) were excluded. The uterotomy incision was always made approximately 1 cm below the deflection of the peritoneum from the bladder onto the anterior wall of the uterus. Pathological examination of hematoxylin–eosin stained sections confirmed the presence of typical endocervical epithelium in all the specimens.

All specimens were excised with scissors from a point in the midline on the lower edge of the uterine incision and contained all layers of the uterine

wall from the perimetrium to the endometrium. After cleaning them of blood and AF, the specimen was divided into three equal portions. The two parts of the tissue which were used for protein extraction and PCR were frozen immediately in nitrogen and stored at -80°C . Tissues for *in situ* hybridization were fixed in 6% neutrally buffered formaldehyde and embedded in paraffin. Hematoxylin–eosin stained slides confirmed the cervical origin of the tissue by the finding of typical endocervical mucosa. In addition anticytokeratin was used as a marker for staining glandular epithelium.

RNA Extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturers instructions. One milliliter of reagent was used. For basic RT-PCR 1 μg of total RNA was subjected to random primed (Invitrogen) cDNA synthesis followed by 30 cycles of PCR in a thermocycler (Applied Biosystems, Foster City, CA) with the use of specific primers for IL-6 and IL-8. The sequences of these primers were published earlier (6,7). The products were analyzed on an ethidium bromide-stained 1.5% agarose gel. The expected fragment length for IL-6 was 639 and 302 bp for the IL-8. Glyceraldehyde phosphate dehydrogenase, a housekeeping gene, was used as an internal control and its fragment size was 248 bp.

In Situ Hybridization

Paraffin sections were cut at 6 μm thickness, collected onto slides precoated with 3-aminopropyltriethoxy-sane, and fixed onto the slides by overnight incubation at 37°C on a slide warmer. *In situ* hybridization was performed using biotinylated oligonucleotide. The same antisense (3') oligonucleotide used for PCR was used as the hybridization probe. The sense strand (5') was used as a negative control for the specificity of hybridization. Sections were deparaffinized and stored in cold ethanol until ready to use. The hybridization solution was prepared by mixing 50% (v/v) deionized formamide, 2 $\times$ Denhardt's solution, 2 $\times$ saline-sodium citrate buffer, 0.1% sodium dodecyl sulfate, 0.1 Mol/L Tris, pH 7.5, and 800 mL of vandydyl ribonucleoside (Life Technologies, Bethesda, MD) complexes. Slides stored in ethanol were washed with hybridization solution and prehybridized in hybridization solution for 1 h at room temperature. Biotinylated probes were added to

the hybridization solution to a final concentration of 0.5 $\mu\text{g/mL}$. Hybridization was done at 45°C for 16 h in a humidified chamber, followed by washing with 0.1 $\times$ saline-sodium citrate buffer at room temperature for 15 min. After blocking endogenous alkaline phosphatase activity, hybridization was detected with use of streptavidin-alkaline phosphatase, followed by incubation with nitroblue tetrazolium-4-bromo-5-chloro-3-indolylphosphate in alkaline buffer solution pH 9.5. Sections were washed in deionized water and mounted with Gelmount (Zymed, San Francisco, CA).

Protein Extraction and Enzyme Linked Immunosorbent Assay

The frozen specimens were homogenized and the protein extracted overnight by a standardized technique using Tris/NaCl (0.02 Mol/L Tris-HCl, pH 8.5 with 0.125 Mol/L NaCl) and proteinase inhibitors (1 mMol/L Di-Isopropyl-fluorophosphate, 10 mMol/L ethylenediaminetetraacetate [Na_2EDTA], 5 $\mu\text{M/L}$ pepstatin, and 50 $\mu\text{M/L}$ E64; 10). The extract was clarified (centrifugation at 100,000 $\times g$ for 1 h at 4°C) and total protein content was measured by the pyrogallol-red method using an automated analyzer (Dimension AR; Dade, Munich, FRG). The concentrations of IL-6 and IL-8 were determined by automated immunoassays (DPC Biermann, Bad Nauheim, FRG), the upper and lower calibration range for IL-6 and IL-8 being 2000–1 pg/mL and 7500–2 pg/mL, respectively. The results were expressed as picograms of cytokine per mg of total protein.

Statistical Evaluation

Statistical evaluation of protein quantitation was done using Wilcoxon's two-sample test. A $P < 0.05$ was considered significant. The in situ slides were evaluated by three observers independent from each other with an inter observer-variability of less than 10%.

RESULTS

All the samples collected were subjected to RT-PCR for IL-8 and IL-6. Specimens from the lower uterine segment at less than 4 cm of dilatation express IL-8 mRNA and did not contain detectable IL-6 mRNA by PCR. At more than 4 cm of dilatation IL-8 mRNA expression was observed in all specimens. IL-6 mRNA was present only in one specimen at

Fig. 1. In situ hybridization from an early stage of cervical dilatation (<4 cm): IL-6 mRNA is localized to a few smooth muscle cells (original magnification $\times 20$).

>6 cm dilatation, whereas IL-8 is positive in all the specimens tested at >6 cm. GAPDH expression (positive control-house keeping gene) was demonstrated in all specimens. There were no detectable changes in the GAPDH expression in any of the samples tested.

In situ hybridization with the sense strand (control) showed negative results in all tissues examined. In early stages of cervical dilatation (<4 cm) IL-6 (Fig. 1) and IL-8 mRNAs were localized to a few smooth muscle and inflammatory cells. With advanced dilatation (>4 cm) an intense staining was documented in the glandular epithelium (Fig. 2), which became obvious around 4 cm. Staining was also observed in the muscle layer of the vessels (data not shown). These

Fig. 2. In situ hybridization from an advanced stage of cervical dilatation (>4 cm): Intensely stained IL-8 mRNA can be detected in the glandular epithelium (original magnification $\times 40$).

Table I. IL-6 Concentration in Tissues

Datation	N	Median	Range	P
<2	8	12.6	3.3–491	
2 to <4	9	91.9	4.6–7295	ns (vs. <2 cm)
4–6	10	1226.2	21.6–8760	<0.05 (vs. <2 cm)
>6	9	1170.7	169.7–4222	<0.05 (vs. <2 cm)

Note. The concentrations are expressed in pg/mg of total protein.

experiments were repeated in all tissue segments to confirm the data.

ELISA documented the protein levels in tissue homogenates. The median values of protein levels are expressed in picograms/mg of total proteins. The median IL-6 protein concentration in the lower uterine segment rose from 12.6 (range 3.3–491 pg/mg) at <2 cm, through 91.9 pg/mg (range 4.6–7295 pg/mg) at 2 to <4 cm, to 1226 pg/mg (range 21.6–8760 pg/mg) at 4–6 cm. A slight drop in the levels at >6 cm was not significant 1171 pg/mg (range 169.7–4222 pg/mg) (Table I). The median IL-8 protein concentration increased from 17.2 pg/mg (range 2–649 pg/mg) at <2 cm and 26.5 pg/mg (range 2.6–11677 pg/mg) at 2 to <4 cm to 2080.7 pg/mg (range 4.4–12450 pg/mg) at 4–6 cm. The median value at >6 cm was slightly lower (1627 pg/mg; range 218–6336 pg/mg), but both this and the value for 4–6 cm cervical dilatation were statistically significantly higher than at <2 cm ($P < 0.05$) (Table II).

DISCUSSION

The roles of inflammatory cytokines (IL-6 and IL-8) have been studied extensively in the context of infection and preterm labor. IL-6 and IL-8 expression has been documented in gestational tissues (decidua, chorion, and amnion) (6,11). IL-6 and IL-8 mRNA were found to be rare in tissues from nonlaboring patients but was detected in almost all tissues after labor (regardless of infection). Saito *et al.* (12) localized IL-8 mRNA and protein in decidual stromal cells, decidual lymphocytes, and endometrial glands. However, no

data exist to show their role in cervical dilatation. This study examined the expression pattern of inflammatory cytokine genes and to relate them to the protein concentrations in the lower uterine segment during parturition at term. At less than 4 cm of dilatation the low IL-6 and IL-8 mRNA expression correlated with low levels of protein concentrations; at more than 4 cm of dilatation high mRNA expression correlated with high levels of IL-6 and IL-8 protein concentrations.

This study also offers an alternate explanation for the elevated AF levels of these cytokines in the forebag in advanced labor observed by MacDonald *et al.* (13,14). If the cervical glands actively secrete these proteins in the normal course of cervical change, an infection is not necessary for the generation of the elevated forebag cytokine levels late in labor in every pregnancy. Increased expression and production of inflammatory cytokines during the dilatation process also suggest that the increased AF concentrations of these cytokines are associated with the dilatation process and not an aftereffect of labor.

In our study the IL-6 expression demonstrated by PCR was somewhat inconsistent. Similarly, Zhong *et al.* (15) did not observe a precise correlation between IL-6 mRNA expression and protein secretion in lipopolysaccharide-stimulated macrophages. The message for IL-6 was only observed for a short time after a high dose and a long time of lipopolysaccharide stimulation. This study did not examine the regional differences in cytokine production by cervical tissues (i.e. uterine tissue near cervix or uterine apex).

In summary our results suggest that increased cytokine expression and release into the lower uterine segment during parturition is associated with cervical effacement. It is also consistent with the clinical observation of the active phase of labor starting around a dilatation of 4 cm as the cervix nears complete effacement (16). Probably the regulation of IL-6 and IL-8 in the human uterine cervix during parturition takes place at the transcriptional level.

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Note. The concentrations are expressed in pg/mg of total protein.

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