

## Parturition at term: parallel increases in interleukin-8 and proteinase concentrations and neutrophil count in the lower uterine segment

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**A relationship was sought between the tissue concentrations of interleukin (IL)-8, matrix metalloproteinase (MMP)-8 and MMP-9, and the numbers of the various leukocytes infiltrating the lower uterine segment stroma during parturition. Biopsy specimens of the lower uterine segment were obtained from 63 women undergoing Caesarean section at various stages of cervical dilatation at term. The concentrations of IL-8, MMP-8 and MMP-9 were determined with enzyme-linked immunosorbent assays, and the leukocytes were quantified immunohistochemically. The median IL-8 concentration (pg/mg total protein) rose significantly from 17.2 at <2 cm dilatation, to 26.5 at 2 to <4 cm dilatation, and 1954.0 at 4–6 cm dilatation, and remained at approximately this concentration at >6 cm dilatation. The median MMP-8 concentration (ng/mg total protein) increased significantly from 32.2 at <2 cm dilatation to 114.2 at >6 cm dilatation. The median MMP-9 concentration (ng/mg total protein) rose significantly from 15.4 at <2 cm dilatation to 102.1 at >6 cm dilatation. The number of neutrophils was significantly higher at 4–6 cm and >6 cm dilatation than at >2 cm, reaching maximum values at >6 cm dilatation. The findings in this study support the hypothesis that IL-8-induced infiltration of the cervical stroma by neutrophils and subsequent release of proteinases may play a key role in parturition.**

**Key words:** cervix uteri/interleukin-8/matrix metalloproteinases/neutrophils/parturition

### Introduction

In contrast to the ripening of the cervix that occurs slowly during pregnancy and is characterized by a decrease in the collagen concentration to 30% and of the proteoglycan concentration to 50% of the non-pregnant state, the dilatation of the cervix that takes place within a few hours during parturition necessitates, in addition to other processes [e.g. rearrangement

or opening up of the collagen helical structure (Uldbjerg *et al.*, 1983; Aspden, 1988; Yu and Leppert, 1991)], the action of catabolic enzymes that degrade elementary structural proteins (e.g. collagens and proteoglycans) in the extracellular matrix of the cervical connective tissue (Rath *et al.*, 1994). The findings of in-vitro experiments on animal tissue suggest that the collagenases derive from the stromal cells (for review see Hulboy *et al.*, 1997). However, neutrophils are thought to be the main source of these enzymes in humans (Osmers *et al.*, 1992) as they are found in increased numbers in the cervical stroma during parturition (Junqueira *et al.*, 1980; Bokstrom *et al.*, 1997). Migration of these cells out of the blood vessels requires a temporary increase in adhesiveness of the endothelium, which is mediated by cell adhesion molecules (Jutila, 1992). We have found an increase in the expression of endothelial leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1 with increasing cervical dilatation (Winkler *et al.*, 1998).

Interleukin (IL)-8 is a cytokine that has a chemotactic effect on neutrophils (Peveri *et al.*, 1988) and also stimulates activation and degranulation of these cells, thus provoking the release of proteolytic enzymes (Willems *et al.*, 1989). Human cervical tissue has been shown to produce IL-8 (Barclay *et al.*, 1993).

This study was undertaken to determine the nature and number of leukocytes infiltrating the stroma of the lower uterine segment during cervical dilatation, and to look for a relationship with the tissue concentrations of IL-8, matrix metalloproteinase (MMP)-8 and MMP-9.

### Materials and methods

#### Tissue specimens

Biopsy specimens were taken from the lower uterine segment, which is considered representative of cervical tissue (Rajabi *et al.*, 1988), of 63 women with singleton pregnancies undergoing non-elective Caesarean section at term (38–42 weeks gestation). The cervix was dilated <2 cm in 18 women, 2 to <4 cm in 15 women, 4–6 cm in 14 women, and >6 cm (6–10 cm) in 16 women, as established by vaginal examination performed between contractions immediately before operation. All the women in the study had been experiencing contractions prior to Caesarean section, including those at <2 cm cervical dilatation. The uterine incision was made ~1 cm below the deflection of the peritoneum from the bladder onto the anterior wall of the uterus. The specimens were excised with scissors by a standardized procedure from a point in the midline on the lower edge of the uterine incision, and were later confirmed to consist of cervical tissue by the finding of typical endocervical mucosa in haematoxylin and eosin-stained sections. The specimens were irrigated with 0.9% NaCl solution to remove blood and amniotic fluid. Tissue to be

**Table I.** Antibodies applied in the study

Antibody against	Main specificity	Dilution	Source
CD3 <sup>a</sup>	T cells	1:100	1
CD20 (L26) <sup>a</sup>	B cells	1:200	2
CD56 <sup>a</sup>	NK cells	1:200	3
CD57 <sup>a</sup>	NK cells	1:80	4
CD68 (KP1) <sup>a</sup>	Macrophages	1:100	2
Tryptase <sup>b</sup>	Mast cells	1:100	2
Vs38c <sup>a</sup>	Plasma cells	1:30	2

<sup>a</sup>Rehydrated sections treated with microwave irradiation (650 W, 3×5 min) in citrate buffer (0.01 M, pH 6.0).

<sup>b</sup>Rehydrated sections exposed to 0.1% pronase (Sigma, Munich, Germany) for 7 min at 37°C.

1: Novocastra, Newcastle, UK; 2: DAKO, Glostrup, Denmark; 3: Monosan, Uden, Netherlands; 4: Becton Dickinson, Mountain View, CA, USA.

NK = natural killer; T = thymus-derived; B = bone marrow-derived.

used for investigation of the IL-8 concentration was then frozen immediately in liquid nitrogen and stored at -80°C until processed. Tissue to be used for the immunohistochemical investigation of leukocyte numbers was fixed in 2% formaldehyde/2% glutaraldehyde and embedded in paraffin. Patients with clinical or histological evidence of chorioamnionitis or cervicitis were excluded from the study, which had the approval of the local ethics committee.

#### Protein extraction

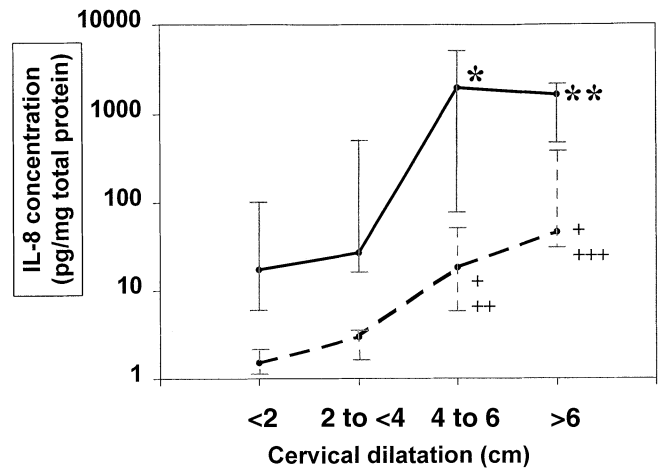
Specimens of frozen tissue (~100–200 mg wet weight) were homogenized for 1 min (Dismembrator; Braun, Melsungen, Germany) and extracted overnight at 4°C in Tris/NaCl (0.02 mol/l Tris-HCl, pH 8.5, with 0.125 mol/l NaCl) containing protease inhibitors [1 mmol/l diisopropyl fluorophosphate, 10 mmol/l EDTA (disodium salt), 5 µmol/l pepstatin, and 50 µmol/l E64]. All chemicals were obtained from Sigma (Munich, Germany).

#### IL-8, MMP-8 and MMP-9 assays

The IL-8, MMP-8 and MMP-9 concentrations were determined in aliquots of the clarified extract (100 000 g, 45 min, 4°C). The IL-8 concentration was determined by an enzyme-linked immunosorbent assay (ELISA; human IL-8 Quantikine, R&D Systems, Abingdon, UK) using a monoclonal antibody specific for both natural and recombinant human IL-8, as described elsewhere (Osmers *et al.*, 1995). The inter- and intra-assay coefficients of variation (CV) were between 5% and 10%. The IL-8 ELISA was performed using a zero standard and seven standard concentrations (31.5–2000 pg/ml), each analysis being performed in triplicate. MMP-8 and MMP-9 concentrations were determined by a sandwich ELISA described by Bergmann *et al.* (1989). The inter- and intra-assay CV were between 2% and 5%. The MMP-8 and MMP-9 assays were performed using standard concentrations of the latent proenzymes. The sensitivities of the MMP-8 and MMP-9 assays were 0.94–25.00 ng/ml and 0.38–25.00 ng/ml respectively. Each analysis was performed in triplicate. The total protein (total protein) concentration was determined by the pyrogallol method using a Dimension assay (Dade, Munich, Germany).

#### Investigation of leukocytes

Paraffin sections were cut at 5 µm, rehydrated and stained with Leder's stain (naphthol AS-D chloroacetate esterase reaction; Leder, 1964) for neutrophils and mast cells and immunostained by the avidin-biotin-peroxidase complex method (Hsu *et al.*, 1981) for mast cells (tryptase), macrophages (CD68), plasma cells (VS38c) and various lymphocyte subsets (CD3, CD20 and CD57). Details of the antibodies applied are given in Table I. The number of each of the



**Figure 1.** Interleukin-8 (IL-8) concentrations (solid line) and number of stromal neutrophils (broken line) in the lower uterine segment during parturition at term. The data are represented on a log scale. Medians and 25th/75th percentiles are given. \* $P < 0.05$ , \*\* $P < 0.01$ , + $P < 0.001$  compared to <2 cm; +++ $P < 0.05$ , ++++ $P < 0.01$  compared to 2 to <4 cm. Pearson's correlation coefficient: 0.15.

various cell types infiltrating the stroma of the lower uterine segment was determined light microscopically by two independent observers (M.W. and T.M.), who evaluated the number of stained cells in the stroma in each of five randomly selected fields using an eyepiece with a counting grid at a magnification of ×250. In cases where the number of these cells was very small, the whole section was evaluated. The number of cells per mm<sup>2</sup> was then calculated, and the average of the values obtained by the two observers was recorded. Interobserver variability was between 10% and 150%; it was especially high in cases with very low density of neutrophils (<20/mm<sup>2</sup>).

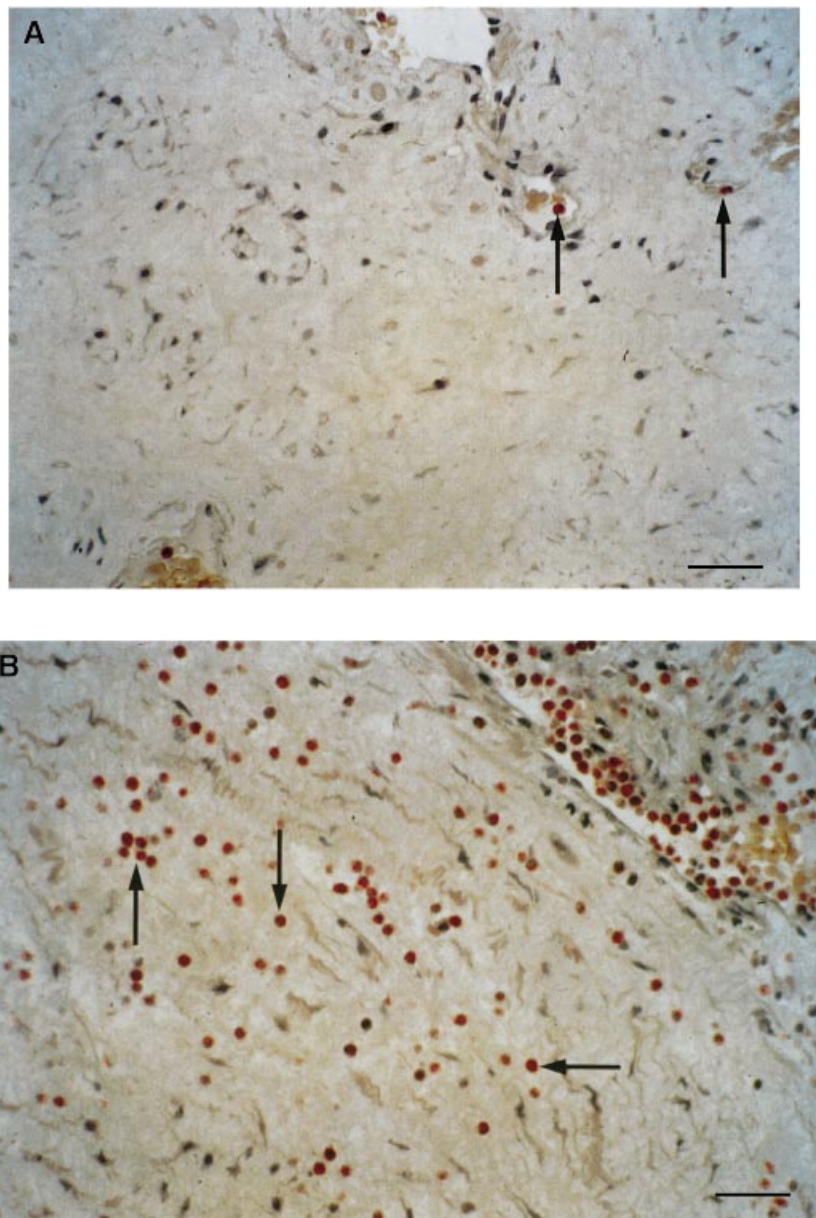
#### Statistical analysis

The Wilcoxon rank sum test was used to analyse differences in the numbers of leukocytes and IL-8, MMP-8 and MMP-9 concentrations at the various stages of cervical dilatation. A probability value of < 0.05 was considered significant. Relationships between the various parameters were sought by correlation analysis and determination of the Pearson's correlation coefficient.

#### Results

The median IL-8 concentration increased significantly ( $P < 0.05$ ) from 17.2 (range 2.0–649.6) pg/mg total protein at <2 cm cervical dilatation to 26.5 (range 2.6–2403.7) pg/mg total protein at 2 to <4 cm dilatation and 1954.0 (range 4.4–12449.5) pg/mg total protein at 4–6 cm dilatation, and remained high (1626.9 pg/mg total protein, range 217.5–6336.7 pg/mg total protein) at >6 cm dilatation (Figure 1).

Only occasional neutrophils (median <1/mm<sup>2</sup>) were found in the stroma of the lower uterine segment at <2 cm dilatation (Figure 2). The median numbers at 4–6 cm (18/mm<sup>2</sup>) and >6 cm (46/mm<sup>2</sup>) were significantly higher ( $P < 0.001$ ; Figures 1 and 2). Neutrophils could easily be distinguished from mast cells, the only other cell population that was positive in Leder's stain, by their typical nuclear morphology. The mast cell count was lower (3–5/mm<sup>2</sup>) and independent of cervical dilatation. The other cell types investigated (macrophages, plasma cells and lymphocyte subsets) were present in even smaller numbers



**Figure 2.** Identification of neutrophils by Leder's stain (naphthol AS-D chloroacetate esterase reaction).  $\times 225$ . (A) Lower uterine segment at 2 to  $<4$  cm cervical dilatation. Neutrophils are seen only in small numbers, and are all intravascular (arrows). None are seen in the stroma. (B) Lower uterine segment at  $>6$  cm cervical dilatation. Numerous neutrophils are seen in both the blood vessels and stroma (arrows). Scale bar =  $40 \mu\text{m}$ .

that were also independent of cervical dilatation (data not shown).

The median MMP-8 concentration increased from 32.2 (range 16.7–93.0) ng/mg total protein at  $<2$  cm dilatation to 64.3 (range 33.0–115.6) ng/mg total protein at 2 to  $<4$  cm, 97.1 (range 0.65–482.9) ng/mg total protein at 4–6 cm and 114.2 (range 34.9–408.8) ng/mg total protein at  $>6$  cm. The difference between the concentrations at  $<2$  cm and  $>6$  cm was statistically significant (Figure 3).

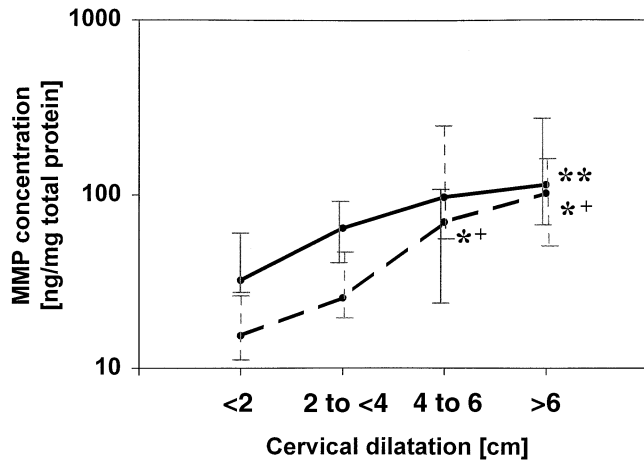
The MMP-9 concentration also increased significantly with cervical dilatation. The median concentration at  $<2$  cm dilatation was 15.4 (range 3.3–51.7) ng/mg total protein, rising to 25.4 (range 13.8–59.8) ng/mg total protein at 2 to  $<4$  cm dilatation, 69.8 (range 8.7–280.5) ng/mg total protein at 4–

6 cm dilatation, to 102.1 ng/mg total protein (range: 35.5–184.1 ng/mg total protein) at  $>6$  cm dilatation (Figure 3).

A good correlation was found between the concentrations of MMP-8 and MMP-9 (Pearson's correlation coefficient: 0.63), and IL-8 and MMP-9 (Pearson's correlation coefficient: 0.63), a weaker correlation between the concentrations of IL-8 and MMP-8 (Pearson's correlation coefficient: 0.33), and no correlation between the number of stromal neutrophils and the concentrations of IL-8, MMP-8 or MMP-9 (Pearson's correlation coefficients  $<0.2$ ).

### Discussion

This study is the first of its kind to describe parallel increases in the concentrations of IL-8, MMP-8 and MMP-9 and the



**Figure 3.** Matrix metalloproteinase (MMP)-8 (solid line) and MMP-9 (broken line) concentrations in the lower uterine segment during parturition at term. The data are represented on a log scale. Medians and 25th/75th percentiles are given. \* $P < 0.01$ , \*\* $P < 0.05$  compared to  $<2$  cm, + $P < 0.05$  compared to 2 to  $<4$  cm. Pearson's correlation coefficient: 0.63.

number of stromal neutrophils in the lower uterine segment during labour at term, as measured in specimens taken from identical sites at four different stages of cervical dilatation.

The concentration of IL-8 in the lower uterine segment rose significantly up to the stage of 4–6 cm dilatation. The finding that there is a significant increase in the concentration of this cytokine in amniotic fluid during term labour (Romero *et al.*, 1991) also suggests that it plays an important role in the process of parturition. Cells of the human trophoblast, decidua, amnion and chorion have been shown to release IL-8 *in vitro* (Saito *et al.*, 1993; Fortunato *et al.*, 1995), and synthesis by amnion and chorion cells has been demonstrated with reverse transcriptase–polymerase chain reaction (Fortunato *et al.*, 1995). In addition to these findings, which suggest a relationship between the rise in IL-8 concentration and the onset of labour, there is other evidence that this cytokine plays a decisive role in cervical dilatation. Local administration of IL-8 in the rabbit and guinea-pig has been found to lead to cervical ripening, with a significant increase in extensibility (Chwalisz *et al.*, 1994; El Maradny *et al.*, 1995), a decrease in collagen content and an increase in water and glycosaminoglycan content of the cervix, and increased infiltration of the cervical stroma by neutrophils (El Maradny *et al.*, 1994). The findings of this study are consistent with those of Osmer *et al.* (Osmer *et al.*, 1995), who found a significant increase in IL-8 concentration in the myometrium of the lower uterine segment during labour, and suggest that IL-8 is also involved in the process of cervical dilatation during parturition. Recently, Sennstrom *et al.* (1997) described an increase in IL-8 concentration in specimens obtained transvaginally from the anterior lip of the cervix of pregnant women at term before the onset of labour, compared with the concentrations in non-pregnant women, and a further increase after vaginal delivery. The finding in this study that the significant increase in IL-8 concentration continues only up to the stage of 4–6 cm dilatation is new. IL-8 concentrations at  $>6$  cm dilatation were the same as at 4–6 cm, or slightly lower. Together with the

morphological findings described here, this suggests that IL-8-induced leukocyte chemotaxis ceases to increase during the later stages of cervical dilatation.

The number of neutrophils invading the stroma of the human lower uterine segment was evaluated for the first time in this study and compared with changes in the concentrations of IL-8, MMP-8 and MMP-9. The greatest numbers of neutrophils were found at  $>6$  cm dilatation. As long ago as 1980, Junqueira *et al.* reported increased infiltration of the cervical stroma by neutrophils during parturition. The presence of a fibre-free 'halo' and increased amounts of amorphous ground substance in the vicinity of these granulocytes, and the positive correlation between the number of infiltrating cells and the degree of collagenolysis seen in many cases were taken as evidence of the release of proteolytic enzymes by these cells. The immunohistochemical results in this study are consistent with these findings. There was also increasing degranulation of the leukocytes up to 6 cm dilatation (Osmer *et al.*, 1992). These observations underline the crucial role of neutrophils, not only in normal and prostaglandin-induced cervical ripening (Greer *et al.*, 1992), but also in cervical dilatation during parturition at term.

Animal experiments using immunohistochemical techniques have revealed an increase in mast cell numbers in the cervix during parturition (Spanggaard *et al.*, 1997). In this study, the number of mast cells and other leukocytes was not found to be related to the degree of cervical dilatation, so it is probable that these cells play only a minor role, if any, in this process in humans.

Also increases were observed in the concentrations of the granulocyte proteases MMP-8 and MMP-9 with cervical dilatation, which is consistent with other published findings (Rath *et al.*, 1987; Rajabi *et al.*, 1988; Osmer *et al.*, 1992; Rechberger and Woessner, 1993). These increases occurred parallel to the invasion of the lower uterine segment stroma by granulocytes. Unlike the IL-8 concentration, these parameters continued to increase after the stage of 4–6 cm dilatation. The levelling off of the IL-8 concentration may suggest that there is strict temporal limitation of the degradative processes in the cervix to prevent more profound tissue damage and enable rapid regeneration of cervical structure after parturition.

The specimens investigated in this study were obtained from the lower border of the uterine incision at Caesarean section. They therefore derived from the lower uterine segment, and not the cervix. Even without the ethical problems that make transvaginal biopsy of the vaginal part of the cervix in pregnancy unjustifiable, it would be difficult to obtain enough tissue by this route to investigate all the parameters of interest here. By measuring collagenase activity, Rajabi *et al.* showed that the same changes occur in both the lower uterine segment and the cervix during parturition (Rajabi *et al.*, 1988). The composition of the lower uterine segment (i.e. the cranial part of the cervix) is not, of course, the same as that of the more caudal parts of the cervix (there is, for example, proportionately less muscle), but we believe that the changes occurring in the lower uterine segment during parturition can, with some reservation, be taken to reflect those occurring in the cervix.

The findings of this study can therefore be considered to

support the hypothesis that the increase in IL-8 concentration and the invasion of the stroma by neutrophils, with the subsequent release of proteolytic enzymes, play a significant role in the process of cervical dilatation during parturition.

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