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Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term

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Accumulating evidence suggests that human parturition represents an inflammatory process. Leukocytes are known to infiltrate uterine tissues but the exact timing, nature and quantity of these cells has not been formally characterized. We have previously demonstrated an apparent increase in pro-inflammatory cytokines within tissues of the labouring uterus. The aims of this study were to quantify and compare the leukocyte subpopulations before and during labour in fetal membranes, decidua and cervix and to quantify and compare mRNA expression of interleukin-1β (IL-1β), IL-6, IL-8 and tumour necrosis factor-α in myometrium, cervix, chorio-decidua and amnion. Biopsies of each of these tissues were obtained from pregnant women delivered by Caesarean section before and after the onset of spontaneous labour at term. Subpopulations of leukocytes were identified using immunohistochemistry and cytokine mRNA expression was quantified using Northern analysis. We found that parturition was associated with a significant increase in IL-1β, IL-6 and IL-8 mRNA expression in cervix and myometrium, IL-6 and IL-8 mRNA expression in chorio-decidua and IL-1β and IL-8 mRNA expression in amnion. Histological analysis demonstrated that leukocytes (predominantly neutrophils and macrophages) infiltrate the uterine cervix coincident with the onset of labour. These data lend further support to the hypothesis that labour is an inflammatory process.

Key words: cervix/cytokines/leukocytes/labour/uterus

Introduction

There is mounting evidence that human parturition represents an inflammatory response (Bowen *et al*., 2002). We and others have shown that leukocytes infiltrate uterine tissues at or around the time of parturition. In the myometrium, there is a massive influx of macrophages, neutrophils and T-lymphocytes coincident with the onset of labour at term (Thomson *et al*., 1999). An influx of inflammatory cells has also been observed in decidua after the onset of labour (Keski-Nisula *et al*., 2000). Although neutrophils and macrophages appear to infiltrate the uterine cervix, the timing of this event differs from that in the myometrium. In the cervix, leukocytic infiltration occurs prior to the onset of labour at term, with no further influx of inflammatory cells demonstrated following the onset of labour (Bokstrom *et al*., 1997).

The function of the leukocytic infiltrate in the process of parturition is unclear. We have previously shown that leukocytes in the myometrium, cervix, decidua and membranes express pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6 and IL-8 (Young *et al*., 2002). Additionally, there appears to be a greater concentration of inflammatory cells in labouring versus non-labouring tissues, although we did not formally characterize this. Others have previously shown up-regulation of pro-inflammatory cytokines in amniotic fluid and in gestational tissues in association with labour (Bowen *et al*., 2002). We hypothesized that expression of messenger RNA of each of the cytokines IL-1β, IL-6, IL-8 and tumour necrosis factor (TNF-α) would be up-regulated in myometrium, cervix and fetal membranes during labour. In association with this, we also hypothesized that the cervix, fetal membranes and decidua are infiltrated by leukocytes during labour, as we have previously shown in the myometrium.

The aims of the present study were (i) to quantify and compare leukocyte subpopulations before and during labour in fetal membranes, decidua and cervix and (ii) to quantify and compare mRNA expression of IL-1β, IL-6, IL-8 and TNF-α in myometrium, amnion, chorio-decidua and cervix before and during labour.

Materials and methods

Subjects

Biopsies of fetal membranes, maternal decidua, cervix and myometrium were obtained from pregnant women delivered by lower segment Caesarean section at term (>37 weeks gestation) (i) during spontaneous labour and (ii) prior to the onset of labour. Labouring women had a cervical dilatation of between 4 and 8 cm. Women with clinical evidence of infection, multiple pregnancy or dysfunctional labour and those who had received prostaglandin or artificial oxytocin were excluded. Written informed consent was obtained from each woman prior to recruitment and the study was approved by the Local Research Ethics Committee.

The cervical specimens were obtained using a scalpel from the anterior lip of the cervix via the uterine incision following delivery of the infant in the labouring group and prior to delivery from the same site *per vaginam* in the non-labouring group, as we have previously described (Ledingham *et al*., 2000). Biopsies of fetal membranes (amnion and chorio-decidua) were sampled from the rupture site (the zone of altered morphology; Malak *et al*., 1994) in

group (i) and from the portion of fetal membranes overlying the uterine cervix in group (ii). Myometrial biopsies were obtained from the upper margin of the lower segment uterine incision during Caesarean section, as we have previously described (Thomson *et al*., 1999). All tissue samples were either fixed in 10% buffered formalin (BDH, Poole, UK) and embedded in paraffin or snap-frozen in liquid nitrogen and stored at –70°C for total RNA extraction.

Immunohistochemistry

Leukocytes and subpopulations of neutrophils, macrophages, B-lymphocytes and T-lymphocytes were identified using primary antibodies (Dako Ltd, Glostrup, Denmark) directed against CD45 (the common leukocyte antigen), neutrophil elastase, CD68, CD20 and CD3 (Table I) respectively in biopsies of amnion, chorio-decidua and cervix both before and during labour. The techniques used were identical to those which we have previously employed to investigate leukocyte subpopulations in myometrium (Thomson *et al*., 1999). Paraffin-embedded tissue sections 5 μm thick were mounted on silane-coated slides and heated to 60°C for 35 min ($n = 10$ for each tissue obtained prior to the onset of labour except for cervix where $n = 8$, and $n = 10$ for each tissue obtained after the onset of labour except for cervix where $n = 8$). Tissue sections were then deparaffinized using xylene and gradually rehydrated with ethanol. Some sections required pre-treatment to retrieve the antigen (Table I). This was done by microwaving the sections at full power for 4–5 min in citrate buffer (10 mmol/l, pH 6.0), or by enzymatic digestion with a 0.1% (w/v) tryspin (Sigma, Poole, Dorset, UK) solution in Tris buffer (pH 7.6) containing 0.1% (w/v) calcium chloride, for 10 min at room temperature. Preincubation was carried out with 1.5% (w/v) normal horse serum in phosphate-buffered saline (PBS; 10 mmol/l sodium phosphate, pH 7.5, 120 mmol/l sodium chloride) for 30 min at room temperature. Thereafter slides were incubated for 60 min with the primary antibody diluted in 1.5% horse serum, then washed with PBS. Further incubation was carried out with biotinylated anti-mouse immunoglobulin (Vector Laboratories, Peterborough, UK) diluted in 1.5% horse serum and 1.5% normal human serum. Sections were then washed in PBS and incubated with avidin DH/biotinylated horse-radish peroxidase H reagent (Vector) in PBS for 30 min before final washing. The antigen was localized using 1 mg/ml diaminobenzidene tetrahydrochloride (DAB; Sigma), 0.2% H_2O_2 in 50 mmol/l Tris–HCl, pH 7.6, which appeared as a brown end-product. Sections were then counterstained with Harris haematoxylin (Sigma). Negative controls included slides incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 *Aspergillus niger* glucose oxidase (Dako), an enzyme that is neither present nor inducible in mammalian tissues. Tonsillar tissue was used as positive control for all antibodies used.

Quantification of inflammatory cell density

Following immunohistochemistry, the inflammatory cells were identified by histological analysis. The number of cell transects in ten randomly selected high power fields (×400 objective magnification) was quantified by two independent observers (I.O. and A.Y.) for each specimen. The area for each high power field was 0.23 mm^2. Both observers were blinded as to whether the tissues were from a labouring or non-labouring source. Inflammatory cells within the blood vessels were not included in the counts. The median density of positive cells for each specimen was calculated. Within specimens of chorio-decidua, leukocyte subpopulations were quantified in chorion and decidua.

Northern analysis

Total RNA was extracted from amnion, chorio-decidua, cervical and myometrial samples ($n = 6$ in each group except for cervix where $n = 4$) using the Trizol® method and quantified using Northern blotting. In view of the difficulties inherent in separating chorion and decidua, these tissues were examined as a single entity, the chorio-decidua (Benirschke and Kaufmann, 1995). 10 μl of RNA sample loading buffer was added to 10 μg of total RNA and the resulting solution separated onto 1.2% agarose gels. Gels were electrophoresed at 60 V for 2.5 h. RNA was transferred and fixed by UV irradiation onto Hybond-N nylon membranes in 20×sodium saline citrate overnight. The nylon membranes were prehybridized in 12 ml of Ultrahyb (AMS Biotechnology, Oxon, UK) for 1–2 h at 42°C and subsequently hybridized with the DNA probes for IL-1β, IL-6, IL-8, TNF-α and the radiolabelled probe CTP and GAPDH. DNA probes were generated from stimulated ThP1 cells by RT–PCR using oligonucleotide primers. These primers were designed, and their product size and sequence validated using the GenBank database (www.nchi.nlm.nih.gov). After hybridization, the nylon membranes were washed and autoradiography was performed at –70°C for 2–7 days. The intensity of the bands on the autoradiographs for each of the cytokines was compared to the housekeeping gene human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the ratio determined. Stimulated THP1 cells were used as positive controls.

Table I. Primary antibodies used for immunohistochemistry

Antigen	Cell type	Pre-treatment	Dilution
CD45	Leukocytes	Microwave	1:1000
CD68	Macrophages	Trypsin	1:50
Neutrophil elastase	Neutrophils	Nil	1:150
CD20	B-lymphocytes	Microwave	1:50
CD3	T-lymphocytes	Microwave	1:50

RT–PCR

RT–PCR was performed when Northern analysis failed to detect a cytokine message. cDNA was generated from 5 μg of RNA from each of amnion, chorio-decidua, cervix and myometrium in labour ($n = 3$ for each tissue) and not in labour ($n = 3$ for each tissue) using the Superscript Preamplification System for first strand cDNA synthesis (Invitrogen, Paisley, UK). Briefly, 5 μg of RNA was made up to 11 μl with distilled water. 1 μl of Oligo (dT) was added and the sample incubated for 10 min at 70°C. 7 μl of a mix of 2 μl 10×buffer, 2 μl 25 mmol/l $MgCl_2$, 1 μl 10 mmol/l dNTP and 2 μl 0.1 mol/l DTT was added and incubated for 5 min at 42°C. After the addition of 1 μl Superscript II, the sample was incubated for 50 min at 42°C, then 15 min at 70°C. Finally 1 μl RNase H was added and incubated for 20 min.

The oligonucleotide PCR primers (5′ ATGAGCACTGAAAGCATGATC and 3′ TCACAGGGCAATGATCCCAAAGTAGACCTGCCC) used to amplify TNF-α cDNA were designed and validated (for product size and sequence) using the GenBank database (www.nchi.nlm.nih.gov). PCR was carried out on a PCR Express machine (Hybaid, Ashford, UK). Briefly 50–100 ng of template DNA was amplified using 0.5 IU *Taq* polymerase (Advanced Biotechnologies, Surrey, UK) in 1×Buffer IV [(200 mmol/l $(NH_4)_2SO_4$, 750 mmol/l Tris–HCl pH 8.8), 0.1% (v/v) Tween 20 (Bioline, London, UK), 1.5 mmol/l $MgCl_2$ (Bioline), 200 μmol/l dNTP (dATP, dGTP, dTTP, dCTP (Roche Diagnostics)]. 0.12 μmol/l of each primer was made up to a final volume of 12.5 μl with sterile distilled water. Incubation was at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 2 min with a final extension of 72°C for 10 min. A 10 μl aliquot of the PCR product was mixed with 5 μl of loading buffer and run out on a 2% agarose gel containing 1 μl of ethidium bromide in a horizontal gel tank in Tris–acetate electrophoresis buffer with a constant current of ~80 V. The samples were also amplified for the control 18S to check that first strand cDNA synthesis was successful.

Quantification of pro-inflammatory cytokines

Following Northern analysis, the autoradiograph intensity for each cytokine was compared to GAPDH and the ratio was determined using Bio-Rad Multi-Analyst/PC 1.1. The cytokine:GAPDH ratio was compared between labouring and non-labouring groups to investigate changes in mRNA expression during parturition.

Statistical analysis

Statistical differences between groups for each tissue type were explored using the Kruskal–Wallis test for both inflammatory cell density and mRNA quantification with Mann–Whitney *U*-test as a post-hoc test for autoradiograph band intensity. Where cytokine mRNA expression and leukocyte density had been determined in biopsies from the same patient (cervical samples only), the association between mRNA concentration and leukocyte density was explored by calculating the partial correlation coefficient (adjusted for the presence or absence of labour). All statistical analyses were performed using SPSS for Windows, version 8. $P < 0.05$ was considered significant.

Table II. Median (interquartile range) density of inflammatory cells in cervix and decidua before and during labour at term per high power field

	Cervix			Decidua		
	Before labour	After labour	*P*	Before labour	After labour	*P*
CD45	10 (1–15)	22 (14–55)	< 0.04	20 (13–33)	22 (8–36)	NS
CD68	<1 (<1 to <1)	6 (3–11)	< 0.02	2 (<1–4)	1 (<1–2)	NS
Neutrophil elastase	<1 (<1 to <1)	23 (3–96)	< 0.001	<1 (<1–3)	<1 (<1–1)	NS
CD3	6 (4–16)	18 (9–33)	NS	2 (<1–11)	1 (<1–7)	NS
CD20	<1 (<1 to <1)	<1 (<1 to <1)	NS	<1 (<1 to <1)	<1 (<1 to <1)	NS

NS = not significant.

Results

Cervix and myometrium

Cervical biopsies were composed mainly of cervical stroma. There was insufficient epithelial tissue, particularly after microwaving, for formal comparison of leukocyte numbers to be made in this area. Our results are therefore restricted to the cervical stroma. All inflammatory cell types were identified in labouring and non-labouring cervical stroma (Figure 1 and Table II). Total leukocyte density, and the densities of neutrophils and macrophages was greater in labouring versus non-labouring cervix.

mRNA for cytokines IL-1β, IL-6 and IL-8 was identified in labouring and non-labouring myometrium and cervix and was significantly greater following spontaneous labour compared with non-labouring tissues ($P < 0.02$) (Figure 2a and b). TNF-α messenger RNA expression was not detected using Northern analysis but was weakly detected in two out of the six myometrial samples (both obtained from women in labour) and one out of the six cervical samples.

Within cervical tissue, there was a significant correlation between mRNA expression of each of IL-1β, IL-6 and IL-8 and total leukocyte density, after adjusting for the presence or absence of labour [partial correlation coefficients of 0.94 ($P < 0.02$), 0.94 ($P < 0.02$) and 0.92 ($P < 0.03$) respectively].

Amnion and choriodecidua

As with the cervix, we were able to identify inflammatory cells in the amnion and chorio-decidua (Figure 1). The median density of leukocytes, macrophages, neutrophils, T-lymphocytes and B-lymphocytes was 1 or <1 per high powered field in the amnion and chorion with no significant differences after labour compared with before labour. Decidual cell densities are described in Table II. There were significantly greater densities of leukocytes and macrophages in the decidua compared with that in the amnion and chorion ($P < 0.02$). The expression of IL-1β ($P < 0.02$) and IL-8 ($P < 0.01$) mRNA but not of IL-6 mRNA was significantly greater in amnion following spontaneous labour (Figure 2c). The expression of IL-8 ($P < 0.005$) and IL-6 ($P < 0.05$) but not IL-1β mRNA was significantly greater in chorio-decidua (Figure 2d) following spontaneous labour. TNF-α mRNA expression was detected neither by Northern analysis nor by PCR.

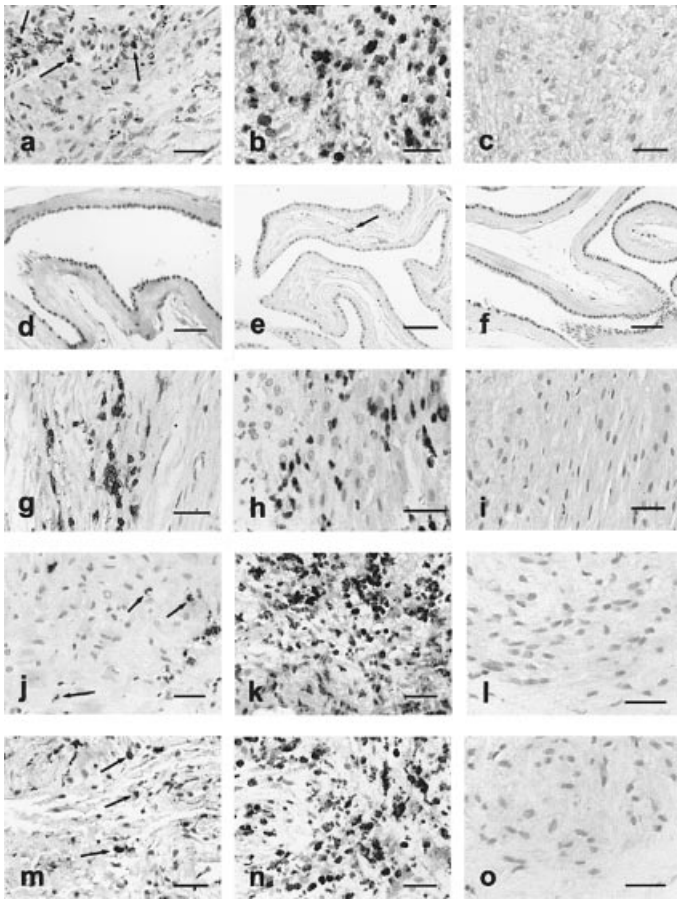

Figure 1. Immunolocalization of leukocytes in human cervix, amnion and chorio-decidua. Total leukocyte density (determined using an antibody against the common leukocyte antigen, CD45) was greater in cervical biopsies collected during labour (**b**) compared with cervical biopsies taken before the onset of labour (**a**). Leukocytes (CD45 positive cells) were sparse in amnion before (**d**) and after (**e**) the onset of labour, but abundant in chorio-decidua before (**g**) and after (**h**) labour. There were no significant changes in leukocyte density in amnion or chorio-decidua following labour. The arrows in (**a**) and (**e**) indicate leukocytes. The density of neutrophils (determined using an antibody against the enzyme neutrophil elastase) was greater in cervix collected during labour (**k**) compared with cervix collected before labour (**j**). The arrows in (**j**) indicate neutrophils. Similarly, the density of macrophages (determined using an antibody directed against CD68) was greater in labouring (**n**) compared with non-labouring (**m**) cervix. The arrows in (**m**) indicate macrophages. The negative control slides (**c**, **f**, **i**, **l** and **o**) showed no staining. Scale bars = 50 μm. The light grey background is haematoxylin counterstain.

Discussion

To our knowledge, this is the first time that peripartum pro-inflammatory events have been assessed in each of the principal tissue types in the uterus using the same experimental protocol. We have shown that the onset of parturition is associated with an increase in pro-inflammatory cytokines in myometrium, cervix, amnion and chorio-decidua. In contrast, we have observed an influx of inflammatory cells in the myometrium (Thomson *et al*., 1999) and cervix only,

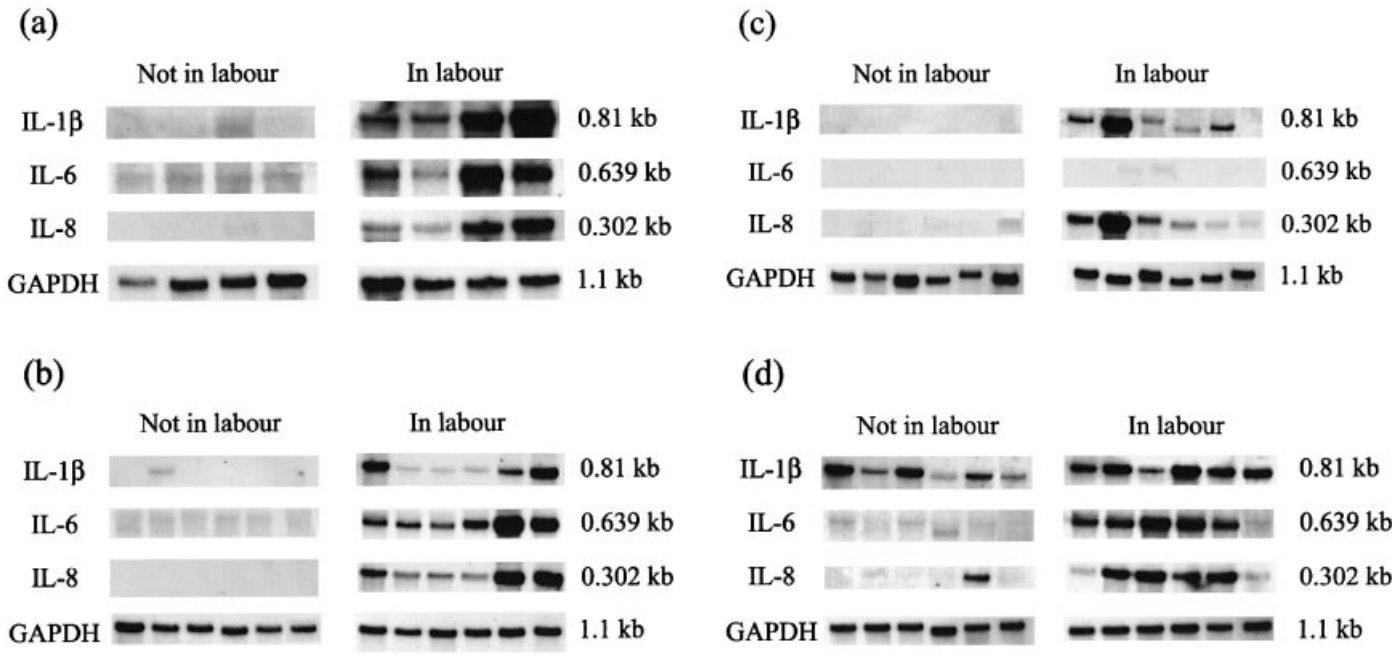

Figure 2. Northern blot hybridization of total RNA (10 μg per lane) from (**a**) cervical samples, (**b**) myometrium, (**c**) amnion and (**d**) chorio-decidua, collected from pregnant women at term before and after the onset of spontaneous labour. The hybridization of each of the pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) was compared with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In cervix (**a**) and myometrium (**b**), the expression of each of IL-1β, IL-6 and IL-8 mRNA was up-regulated following the onset of labour. In amnion (**c**), the expression of IL-1β and IL-8 mRNA was increased following the onset of labour. There was no change in the expression of IL-6 mRNA in amnion with labour. In chorio-decidua (**d**), the expression of IL-6 and IL-8 mRNA was greater in samples collected following the onset of labour compared with samples collected before labour. IL-1β mRNA was detected in chorio-decidua before the onset of labour with no change in expression detected following labour. In each of the tissues examined, TNF-α mRNA expression was below the limit of detection of our assay.

and not in the fetal membranes or decidua, in association with parturition.

In the cervix, leukocytes were sparse prior to the onset of labour, but their density increased significantly in labour in concert with increased cervical production of pro-inflammatory cytokines. Indeed, there were significant correlations between cytokine mRNA expression and leukocyte density within the cervix. In a previous study, we localized each of the pro-inflammatory cytokines to the leukocytes within the cervix, suggesting that invading leukocytes are a major source of the increase in these agents during parturition (Young *et al*., 2002). The work described here confirms previous data showing that the cervix is invaded by leukocytes during the process of cervical ripening and parturition (Junqueira *et al*., 1980; Bokstrom *et al*., 1997). The putative effects of these leukocytes include breakdown and remodelling of cervical tissue via release of matrix metalloproteinases, prostaglandins, cell adhesion molecules and nitric oxide (Thomson *et al*., 1999; Ledingham *et al*., 2000, 2001). The pro-inflammatory cytokines released by the leukocytes and other cell types may also contribute to this process, not least by promoting further leukocyte invasion. The work described here conflicts with previous reports in the precise timing of the leukocytic invasion (Bokstrom *et al*., 1997). In our study, we showed a 2–3-fold increase in leukocyte density in labouring compared with non-labouring tissues. However, Bokstrom *et al.* showed a 2–3-fold increase in leukocyte density from the first trimester of pregnancy to term, prior to the onset of labour. They did not observe any further increase in leukocyte density after the onset of labour. The absolute number of inflammatory cells observed in the labouring samples was similar (when corrected for the area examined) in both that study and the one described here. In our study, we did not take cervical biopsies early in pregnancy or in the non-pregnant state, and cannot therefore be certain that some leukocytic invasion occurred prior to end of pregnancy. Although our methods are apparently similar to those used by Bokstrom *et al.*, it is possible that subtle differences in the characteristics of women recruited have contributed to the discrepant results. For example, Bokstrom *et al.* did not define active labour, whereas we employed strict inclusion criteria, recruiting only those women in spontaneous labour with cervical dilation of 4–8 cm. If the labouring women in the Bokstrom study were in early rather than established labour, an influx of inflammatory cells in their subjects in association with labour might have been masked. If the non-labouring women in our study were more distant from the spontaneous onset of labour (with a less ripe cervix) than the Bokstrom subjects, the rise in leukocyte count which we observed could have occurred in late pregnancy, rather than in labour. We are aware of only one other study which reports leukocyte density in the peripartum cervix (Junqueira *et al*., 1980). This study reports an increase in leukocyte density from non-pregnant cervix to those obtained during parturition (which is consistent both with our results and those of Bokstrom), although quantitative analysis was not performed.

In the myometrium, as in the cervix, parturition is associated with an increase in pro-inflammatory cytokine production, as we have demonstrated in this study, and massive leukocyte invasion, as we have demonstrated in a previous study (Thomson *et al*., 1999). Again, our previously reported data suggest that the invading leukocytes make a significant contribution to pro-inflammatory cytokine produc-

tion. At least one of the cytokines produced, IL-1β, is known to stimulate myometrial contractions both directly and via an increase in prostaglandin production, thus contributing to the fundamental mechanisms of parturition (Hertelendy *et al*., 1993; Molnar *et al*., 1993).

In the fetal membranes and decidua, we observed an increase in pro-inflammatory cytokine production, but failed to show a significant increase in leukocyte density, in association with the onset of labour. There was little IL-6 mRNA expression in amnion either in labour or not in labour. These data are in agreement with those showing amniochorionic IL-6 mRNA expression only when samples were obtained from infected membranes or were stimulated by endotoxin (Menon *et al*., 1995) whereas amniochorionic IL-6 protein expression has been more consistently identified (Laham *et al*., 1996; Keelan *et al*., 1997). In contrast to IL-6, IL-1β was expressed in chorio-decidua before the onset of labour with no significant change after labour. The reasons for this are obscure, but suggest that IL-1β and IL-6 may play differing roles in the chorio-decidua and amnion respectively compared with other tissues within the pregnant uterus. The lack of a change in leukocyte density in the decidua in association with labour is at odds with the results of Keski-Nisula *et al.*, who observed a significant increase in the proportion of tissues showing inflammation after the onset of labour (7/117 specimens before compared with 7/24 specimens after labour). We did observe a trend to an increase in leukocyte cell density after the onset of labour. In retrospect, the range of leukocyte densities in this tissue was wide, and our study was therefore probably underpowered to show anything other than a huge increase in leukocyte density in the decidua.

We did not formally compare cytokine production across tissue types, as this was not the primary aim of our study. However, the cytokine mRNA production from the myometrium is at least as great (per gramme of tissue) as that from the cervix and fetal membranes. Given the signficantly greater mass of the myometrium, if protein synthesis reflects mRNA production, the myometrium will make by far the greatest contribution to pro-inflammatory cytokine production. This contrasts with data indicating that the fetal membranes play a pivotal role in the initiation of parturition via nuclear factor-kappaB activation, synthesis of cyclooxygenase 2 and production of prostaglandins (Slater *et al*., 1999; Allport *et al*., 2001). We hypothesize that the myometrium and fetal membranes play complementary roles during the process of labour—the trigger to parturition may be delivered from the fetal membranes, possibly acting on signals received from the fetus (Challis *et al*., 2000). Leukocyte invasion and pro-inflammatory cytokine production may then be stimulated in the myometrium in a feed forward loop which sustains and amplifies the process of parturition via prostaglandin production and uterine contractions.

In summary, we have shown that parturition is associated with leukocyte invasion and pro-inflammatory cytokine production in the cervix and myometrium. We observed a selective increase in some of the cytokines in the fetal membranes and decidua. These data support the hypothesis that labour is an inflammatory process. If similar processes occur in preterm labour, they may provide novel therapeutic targets for the treatment of this condition.

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