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**Inflammatory Mediators**

**In**

**Parturition**

by

**Anne Young**

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**Thesis submitted for the degree of Master of Science to the**

**University of Glasgow**

**Division of Developmental Medicine**

**Reproductive and Maternal Medicine**

**Glasgow University**

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## Summary

Inflammatory mediators in the cervix, fetal membranes and myometrium play a crucial role in parturition. Each of the pro-inflammatory cytokines interleukin (IL)-1 $\beta$ , IL-6, IL-8 and Tumour Necrosis Factor (TNF) $\alpha$  has been identified in reproductive tissues during labour. The cellular origins of these cytokines is unclear. The aim of this study was to localise the above pro-inflammatory cytokines using immunocytochemistry and quantify pro-inflammatory cytokine expression using northern analysis. These protocols were carried out in cervix, fetal membranes and myometrium at term. Biopsies were taken from women undergoing caesarean section either before or after the onset of labour. The density of leukocytes in these tissues was also determined by immunolocalisation using the following antibodies, CD45 (leukocytes), CD3 (T-cells), CD20 (B-cells), CD68 (macrophages) and neutrophil elastase (neutrophils). In myometrium and cervix, immunostaining for IL-1 $\beta$  was predominantly in leukocytes. In fetal membranes, IL-1 $\beta$  localised to leukocytes and to the stromal cells of the decidua. In myometrium, IL-6, IL-8 and TNF $\alpha$  were restricted to leukocytes which were present in greater numbers in tissue obtained during labour. In cervix, IL-6, IL-8 and TNF $\alpha$  localized to leukocytes, glandular and surface epithelium. IL-8 also localised to cervical stromal cells. In fetal membranes, IL-6 and TNF $\alpha$  were expressed by decidual stromal cells, infiltrating leukocytes and extravillous trophoblast. In membranes, IL-8 localized to leukocytes in the chorion but was not detected in the amnion. In fetal membranes collected in labour, IL-8 was expressed in decidual stromal cells.

In northern analysis the ratio of IL-1 $\beta$ -mRNA/GAPDH mRNA expression was significantly higher in amnion ( $p < 0.02$  [median 0.21 (interquartile range IQ 0.12-0.35)]), myometrium ( $p < 0.02$  [median 0.27 (IQ range 0.12-1.4)]) and cervix ( $p < 0.02$  [median 1.45 (IQ range 0.69-2.43)]) following spontaneous labour compared to not in labour, amnion (median 0 [IQ range

0-0]), myometrium (median 0 [IQ range 0-0]) and cervix (median 0 [IQ range 0-0]). We found the ratio of IL-6 mRNA/GAPDH mRNA significantly increased in chorion ( $p < 0.05$  [median 1.42 (IQ range 1.10-2.06)]), myometrium ( $p < 0.005$  [median 1.34 (IQ range 1.23-2.40)]) and cervix ( $p < 0.05$  [median 1.35 (IQ range 0.65-2.53)]) after the onset of spontaneous labour compared to not in labour, chorion (median 0.28 [IQ range 0.20-0.38]), myometrium (median 0.13 [IQ range 0.09-0.16]) and cervix (median 0.23 [IQ range 0.18-0.31]). The expression of IL-8 was significantly higher in amnion ( $p < 0.01$  [median 0.24 (IQ range 0.10-0.40)]), chorion ( $p < 0.005$  [median 0.89 (IQ range 0.38-1.62)]), myometrium ( $p < 0.005$  [median 0.63 (IQ range 0.50-1.8)]) and cervix ( $p < 0.02$  [median 1.30 (IQ range 0.33-2.4)]) following spontaneous labour compared to not in labour, amnion (median 0 [IQ range 0-0]), chorion (median 0 [IQ range 0-0]), myometrium (median 0 [IQ range 0-0]) and cervix (median 0 [IQ range 0-0]). TNF $\alpha$  expression was below the limits of detection for our assay. The density of leukocytes and of the subfractions, neutrophils, macrophages, T-cells and B-cells in cervix and myometrium were significantly greater in tissues obtained during labour. The density of leukocytes and white cell subfractions were significantly greater in decidua than in either chorion and amnion, but there was no difference in tissues taken either before or after the onset of labour. Establishing which of the pro-inflammatory cytokines are expressed in uterine tissues before and after the onset of labour, and determining the leukocytes involved in this expression, could lead to the development of strategies which could be effectively employed to treat pre-term labour which is a major problem in obstetrics.

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## **Aknowledgements**

I would like to thank my supervisors Dr Dilys Freeman and Professor Ian Greer for their support, advice and encouragement throughout this research. I also acknowledge Dr Andrew Thomson and Dr Maricanne Leddingham's assistance in collecting tissue samples and Dr Jane Norman for all her sound advice. A big thankyou to Mrs Fiona Jordan for all her help with northern analysis, for keeping my spirits up and her wonderful sense of humour. I would also like to express my thanks to Miss Frances McGarry for her help with PCR, Dr Inass Osman for her help with microscope analysis and Dr Richard Fleming in the understanding of statistics. Thanks must go to my youngest son Andrew for all his computer wizardry and my two eldest sons Christopher and Jonathan who didn't think the old dear had it in her. I would also like to say thanks to my Mum, she is very proud of me and I know my Dad would have been. Finally thanks to Dave, my husband and best friend I could not have completed this thesis without you.

## Declaration

I declare that I have contributed to the experimental work described in this thesis, with help from Mrs Fiona Jordan with northern analysis, Miss Frances McGarry with PCR, under the supervision of Dr Dilys Freeman in the Division of Developmental Medicine, Reproductive and Maternal Medicine, Glasgow University. The work presented here is original research and has not been previously submitted for a higher degree. I am the author of the thesis and have consulted the references cited.

Signed



Anne Young

I certify that the experimental work reported in this thesis was performed by Mrs Anne Young at the University of Glasgow and that during the period of study she has fulfilled the conditions of the relevant regulations governing the degree of Master of Science (MSc).

Signed



Dilys Freeman

**Abbreviations**

ACTH	adrenocorticotrophin
ATP	adenosine tri-phosphate
C	centigrade
Ca <sup>2+</sup>	calcium <sup>2+</sup>
CAP	contraction associated protein
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CRH	corticotrophin releasing hormone
CTP	deoxycytidine 5'-triphosphate
Cx43	connexin 43
DAB	diaminobenzidine tetra hydrochloride
dATP	deoxyadenosine tri-phosphate
dCTP	deoxycytidine tri-phosphate
DDW	deionised distilled water
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine tri-phosphate
dil	dilution
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside tri-phosphates
dTTP	deoxythymidine tri-phosphate

DW	distilled water
E2	oestradiol
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF $_{\alpha}$	epidermal growth factor- $\alpha$
ELAM-1	endothelial leukocyte adhesion molecule-1
ELISA	enzyme-linked immunosorbant assay
FACS	fluorescence activated cell sorting
g	gravity
GAGs	glycosaminoglycans
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPR	G-protein-coupled receptor protein family
h	hour
HPA	hypothalamic-pituitary-adrenal
HrIL-1	human recombinant interleukin-1
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
JAK	Janus kinases
kDa	kilodalton
LCS	laser scanning cytometry
LPS	lipopolysaccharide
MCP-1	monocyte chemattractant protein-1
MGSA	melanoma growth stimulatory activity

MIP-2	macrophage inflammatory protein 2
MLCK	myosin light chain kinase
MMPs	matrix metalloproteinases
MOPS	[N-morpholino] propane-sulphonic acid
mRNA	messenger ribonucleic acid
n	number
NAD	nicotinamide adenine nucleotide
NAP-1	neutrophil attractant/activating protein
NAP-2	neutrophil activating protein 2
NF $\kappa$ B	nuclear factor kappa B
NH <sub>4</sub>	ammonium
NK	natural killer
OT	oxytocin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PGDII	prostaglandin dehydrogenase
PGE <sub>2</sub>	prostaglandin E <sub>2</sub>
PGF <sub>1<math>\alpha</math></sub>	prostaglandin F <sub>1<math>\alpha</math></sub>
PGF <sub>2<math>\alpha</math></sub>	prostaglandin F <sub>2<math>\alpha</math></sub>
PGH <sub>2</sub>	prostaglandin H <sub>2</sub>
PGHS	prostaglandin H synthase
PGI <sub>2</sub>	prostacyclin

PLA <sub>2</sub>	phospholipase A <sub>2</sub>
PTHrP	parathyroid hormone related peptide
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RPM	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
secs	seconds
SOCS	suppressors of cytokine signalling
SSC	sodium saline citrate
STAT	signal transducer and activation of transcription
TBX	thromboxane
TE	tris EDTA buffer
TGF <sub>β</sub>	transforming growth factor β
TIMPs	tissue inhibitors of metalloproteinases
TNF <sub>α</sub>	tumour necrosis factor α
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1

## **Chapter 1**

### **Introduction**

## **1:1. Overview of parturition**

### **1:1.1. Labour.**

Labour or parturition is the process of giving birth. Parturition results from a complex interaction of maternal and fetal factors. In women the molecular mechanisms involved in term or pre-term labour (pre-term labour is defined as labour before 37 completed weeks) still remain unclear. Many authorities believe that term and pre-term labour are fundamentally the same processes except for the gestational age at which they occur. Pre-term labour continues to be a major problem in obstetrics, approximately 5-10% of all pregnancies result in pre-term birth, 70% of perinatal mortality is associated with this group and up to 75% of neonatal morbidity. A better understanding of the underlying molecular and biochemical mechanisms operating prior to and during labour may lead to the development of improved diagnostic techniques and therapeutic intervention in pre-term labour.

### **1:1.2. Parturition Phase 0.**

Uterine contractility during pregnancy and parturition can be divided into different phases (Challis *et al* 2000, Lye *et al* 1998). In phase 0 the uterus is maintained in a relatively quiescent state throughout pregnancy by the separate or combined actions of inhibitors such as progesterone, prostacyclin (PGI<sub>2</sub>), relaxin, parathyroid hormone related peptide (PTHrP), nitric oxide and corticotrophin releasing hormone (CRH) (Challis *et al* 2000, Lye *et al* 1998, Liggins *et al* 1994, Norwitz *et al* 1999). These substances act in different ways but their actions result in increased intracellular levels of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP). These nucleotides inhibit intracellular calcium release and reduce the activity of the enzyme myosin light

chain kinase (MLCK) that is required for the shortening of the myofilaments leading to contractions.

### **1:1.3. Parturition Phase 1.**

Before term the uterus undergoes changes in activation and stimulation (Lye *et al* 1989). Phase 1 of parturition is linked to activation of uterine function whereby changes within the uterus will allow the uterus to respond to agents that will cause the myometrium to contract. These changes include induction of a cassette of gene products required for contractions. These contraction associated (CAP) genes include connexin 43 (Cx43) a key component of gap junctions and receptors for stimulatory prostaglandins and oxytocin. Ripening or softening of the cervix begins to occur during this phase.

### **1:1.4. Parturition Phase 2.**

In phase 2 of parturition the activated uterus can be acted upon and stimulated to contract by uterotonins. Uterotonins include stimulatory prostaglandins, oxytocin and corticotrophin-releasing hormone (CRH). Phase 2 of parturition begins with stimulation of the myometrial contractions and dilatation of the cervix and ends with the delivery of the fetus and placenta. Phase 2 also includes the three stages of labour. The first stage begins with the onset of regular, painful contractions and ends when the uterine cervix is fully dilated. The second stage of labour begins at full dilatation of the cervix and ends with complete delivery of the fetus. The third stage begins with completion of fetal expulsion and ends with delivery of the placenta.

### **1:1.5. Parturition Phase 3.**

Phase 3 of parturition includes involution of the uterus and the return to the normal nonpregnant state (Challis *et al* 1994 (a), Challis *et al* 1994 (b), Lye *et al* 1989).

### **1:1.6. The control of parturition.**

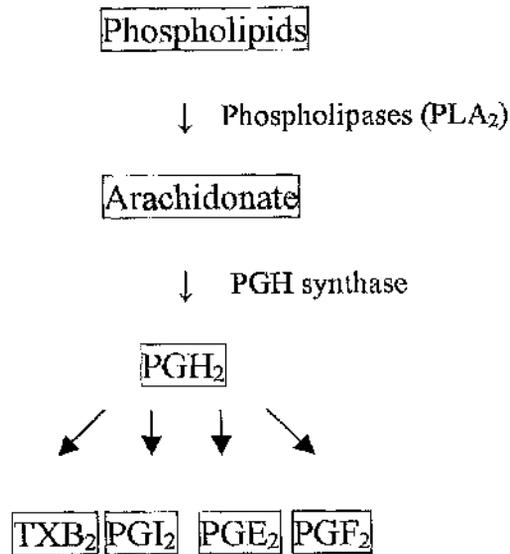
Many factors are involved in controlling the various phases of parturition including oxytocin, prostaglandins, progesterone, oestrogens and corticotrophin-releasing hormone (CRH).

### **1:1.7. Prostaglandins.**

There is evidence that an increase in intrauterine prostaglandin (PG) production adds to the push toward myometrial contractility in both term and preterm labour (Challis *et al* 2000, Kniss 1999). Under normal conditions prostaglandin formation is probably of key importance in the onset of both term and preterm labour in the human. Prostaglandins appear to play a role in phases 1 and 2 of parturition including cervical ripening (Thorburn *et al* 1979). Prostaglandins are not stored but released after synthesis (Bennett *et al* 1967) and are quickly metabolised to biologically less active forms (Samuelsson 1970). Endogenous prostaglandins are formed by enzymatic oxidation and cyclization of certain poly-unsaturated fatty acids. PGE<sub>2</sub> and PGF<sub>2α</sub> are derived from arachidonic acid and PGE<sub>1</sub> and PGF<sub>1α</sub> from di-homo-γ-linolenic acid. To be available as a precursor for prostaglandin biosynthesis arachidonic acid must be in non-esterified (or free) form. Arachidonic acid can arise from a number of intracellular lipid pools such as triglycerides, cholesteryl esters and phospholipids, consequently there are a number of potential acylhydrolases capable of freeing arachidonic acid. Many authors believe the liberation of arachidonic acid to be a major event in the control of prostaglandins and parturition (Gibb 1998, Liggins 1983,

Thiery 1979). The control of prostaglandin biosynthesis for parturition has focussed mainly on the action of two enzymes; phospholipase A<sub>2</sub> and prostaglandin H synthase (PGHS) (Bleasdale 1989, Wilson 1990, Rice 1990). Liberation of arachidonic acid from phospholipids is determined via various phospholipases, in particular phospholipase A<sub>2</sub> (PLA<sub>2</sub>), which has been shown to have two major forms; cPLA<sub>2</sub> (cytoplasmic) and sPLA<sub>2</sub> (secretory). The arachidonic acid formed is then converted to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) via the enzyme prostaglandin H synthase (PGHS). The PGH can then be transformed by various other enzymes to thromboxane (TBX), prostacyclin (PGI<sub>2</sub>), PGE<sub>2</sub> or PGF<sub>2</sub>α. PGHS has been shown to have two isoforms. PGHS-1 is thought to be the constitutive form produced in all cells (Wang *et al* 1993). PGHS-2 is an inducible form of the enzyme whose expression can be upregulated by up to 80-fold in response to various mediators including cytokines and growth factors (Tazawa *et al* 1994, Smith 1992). Regulation of prostaglandin formation transpires at both the phospholipase and PGHS steps. A summary of the biosynthetic pathway for prostaglandin is shown in **Figure 1a**.

**Figure 1a.** Biosynthetic pathway for prostaglandin.

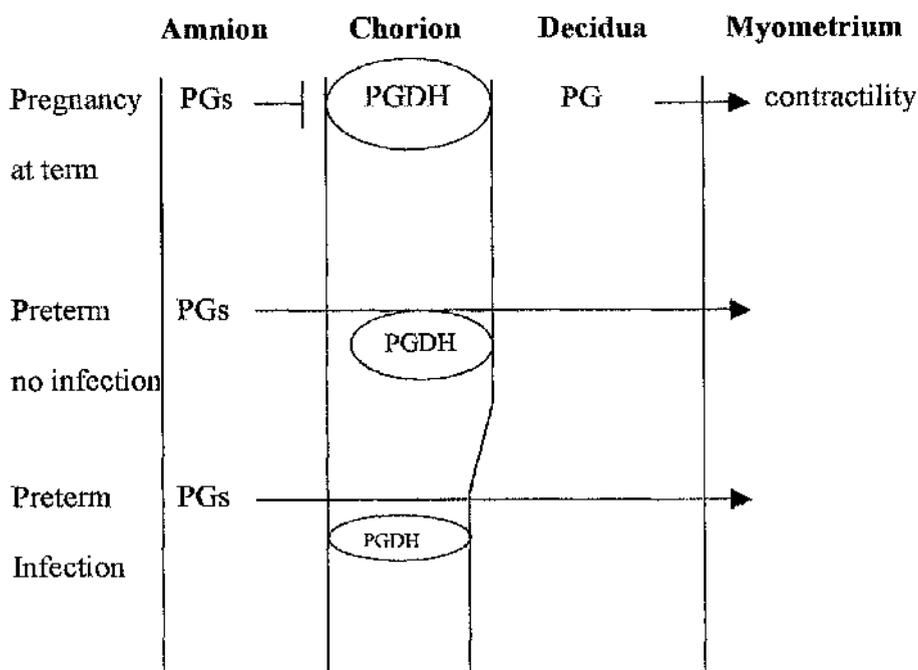


(Based on W.Gibb. The role of prostaglandins in parturition in *Annals Medicine* 1998; 30: 235-241.)

Primary prostaglandins such as PGI<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub> are inactivated through metabolism. The first step involves a nicotinamide adenine nucleotide (NAD<sup>+</sup>)-dependant 15-hydroxy-prostaglandin dehydrogenase (PGDH). The regulation of this enzyme may be significant in the onset of parturition at term. It is known that the important metabolising enzyme for prostaglandins, PGDH, is localised to the trophoblasts of the chorion laeve and acts as a barrier to the transmembrane transport of prostaglandins generated within the amnion and the chorion laeve itself and their passage to the decidua and therefore the myometrium. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), the key prostaglandin produced during active labour, is synthesised in the amnion and has to consequently cross the chorion and decidua to stimulate myometrial contractility. This hypothesis is complicated by the fact that as previously stated chorion contains high 15-hydroxy prostaglandin dehydrogenase activity (Okazaki *et al* 1981) and is thus capable of metabolising PGE<sub>2</sub> to an inactive metabolite during transport from the amnion to the myometrium. Results from *in vivo* studies have shown that transfer of PGE<sub>2</sub> from the amnion to the myometrium occurs despite the

metabolic barrier presented by the chorion (Johnston *et al* 1996). Evidence from immunohistochemical studies (Cheung *et al* 1990) supports this occurrence, studies of the distribution of PGDH in samples of human fetal membranes, decidua and placental tissues confirm the high PGDH concentration of the chorion. This study demonstrated that while some trophoblasts expressed PGDH others lack the enzyme. Thus PGs could be transported across the chorion via the movement of these PGDH deficient trophoblasts. Other researchers have shown that in some cases of idiopathic preterm labour, not associated with infection, there is an obvious reduction in PGDH activity, PGDH mRNA and protein expression within the chorion laeve (Sangha *et al* 1994). They suggest that this decrease may allow prostaglandins produced within the amnion and the chorion laeve to get into the myometrium and cause contractions. Studies have shown that in certain cases of infection with infiltration of the chorion by polymorphonuclear leucocytes, the chorion laeve trophoblasts have been damaged and therefore the PGDH activity of the chorion has been ablated in this process (Van Meir *et al* 1996, Van Meir *et al* 1997a). Additional studies examining the activity of PGDH in various areas of the uterus during normal labour at term have established that there is a decrease in PGDH expression in the fetal membranes in the lower uterine segment covering the cervix, suggesting that this may permit prostaglandins from this area of the membranes to access the cervix and result in cervical ripening (Van Meir 1997b). Alterations may then occur in PGDH activity in the fundal section of the uterus as labour progresses allowing active prostaglandins to reach the myometrium and cause contractions. Below is a diagram (**figure 1b**) to summarize the role of chorionic prostaglandin dehydrogenase (PGDH) in regulating transfer of primary prostaglandins across the human fetal membranes.

Figure 1b.



(Based on Challis and Gibb. Control of Parturition in Prenatal Neonatal Medicine 1 1996: 283-291)

### 1:1.8. Oestrogen and Progesterone.

Oestrogen and progesterone have powerful effects on prostaglandin synthesis in experimental animals (Challis *et al* 1994a), where an increase in oestrogen concentrations and a decrease in progesterone concentrations are thought to be key determinants in causing the increase in prostaglandin synthesis. In humans the situation is less clear. Progesterone concentrations increase continually throughout gestation and oestrogen concentrations increase noticeably during third trimester but also rise over a considerable period of gestation. There have been suggestions that the oestrogen/progesterone ratio may play a role in the regulation of prostaglandins in the human. There is however no evidence for the direct effect of progesterone and/or oestrogen in the regulation of prostaglandin synthesis in human uterine tissues.

### 1:1.9. Glucocorticoids.

In the sheep, it seems that glucocorticoids have a major role in controlling prostaglandin formation (Thorburn *et al* 1979, Liggins 1969). Increases in fetal glucocorticoids act on the sheep placenta to cause a decrease in progesterone and an increase in oestrogen production, this has been implicated in the increase of prostaglandins. PGHS-2 expression increases in sheep placentae with advancing gestation and in glucocorticoid induced labour (McLaren *et al* 1996), and there is an increase in cPLA<sub>2</sub> and PGHS-2 expression in endometrium and myometrium (Zhang *et al* 1996). This process may also occur in the human membranes and placenta. In human amnion cell cultures glucocorticoids have been shown to amplify prostaglandin production by the cultured cells (Gibb *et al* 1990, Mitchell *et al* 1988, Potestio *et al* 1988). This is in contrast to its action on most other systems where glucocorticoids have been shown to inhibit prostaglandin synthesis (DeWitt *et al* 1993). The increase in PG synthesis is due at least in part to increased PGHS-2 expression (Zakar *et al* 1995) and it has been established that glucocorticoids stimulate the fibroblast-like cells in the amnion mesenchyme (Economopoulos *et al* 1996). It is likely, in both ovine and human pregnancy that upregulation of PGHS-2 in the fetal section may in part result from the effects of glucocorticoids. Glucocorticoids increase PGHS expression and prostaglandin formation but also inhibit PGDH activity and therefore decrease prostaglandin metabolism (Patel *et al* 1997). It is therefore possible that glucocorticoids may have a dual role, increasing prostaglandin synthesis via stimulation of PGHS-2 expression and at the same time decreasing prostaglandin metabolism by inhibiting PGDH expression.

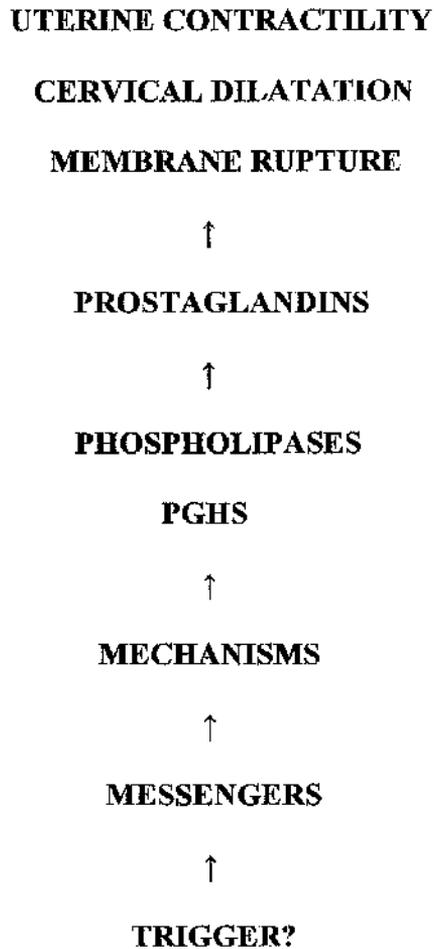
### 1:1.10. CRH and other agents.

In cell culture studies many agents have been found to alter the production of prostaglandins in fetal membranes, these include epidermal growth factor- $\alpha$  (EGF $\alpha$ ), transforming growth factor- $\alpha$  (TGF $\alpha$ ), transforming growth factor- $\beta$  (TGF $\beta$ ) and corticotrophin releasing hormone (CRH). In human pregnancy, the concentration of CRH increases exponentially in maternal peripheral plasma from about 20-24 weeks (Campbell *et al* 1987). There is correlation of CRH concentrations between maternal peripheral plasma and levels in the placenta and in cord blood. The large quantity of CRH mRNA and content of peptide in the human placenta rises increasingly with gestation (Frim *et al* 1988). CRH is also present in human amniotic fluid where its levels are raised significantly in association with preterm labour and intrauterine growth restriction (Warren *et al* 1992, Wolfe *et al* 1988). One of the proposed actions of CRH in inducing labour may be related to the fact that in the human fetal membranes and placenta, glucocorticoids upregulate rather than inhibit CRH expression and that this CRH can cause amplified prostaglandin synthesis resulting in a positive feed-forward effect ending in parturition (Jones *et al* 1989, 1990).

Prostaglandin formation also seems to play an important role in infection induced preterm labour. Studies have shown that interleukins are increased in amniotic fluid in preterm labour with infection and that numerous interleukins can increase prostaglandin production in cultures of amnion, chorion and decidua (Romero *et al* 1991, Mitchell *et al* 1991). These cytokines have been shown to increase the expression of both cPLA<sub>2</sub> and PGHS-2 in cultured tissues (Mitchell *et al* 1993, Xue *et al* 1996). Thus prostaglandin production and action at parturition is the result of complex interaction between many agents.

The Figure 1c. below shows a proposed pathway to parturition.

**Figure 1c.** Based on D.M.Olsen in Prostaglandins and the Uterus Chapter 10.



## **1:2. Cervical Ripening.**

### **1:2.1. The uterine cervix.**

The role of the uterine cervix changes considerably during pregnancy. While it is a rigid and closed structure holding the uterine contents during pregnancy, in the final 6-7 weeks of human gestation the cervix becomes soft, oedematous, thin and compliant allowing it to open easily in response to contractions to facilitate birth. This process is known as cervical ripening and is a vital part of the preparatory changes prior to the onset of labour. The

softening of the cervix in pregnancy is partly due to increases in vascularity and water content but the most striking changes occur in connective tissue. The uterine cervix has little muscle tissue. The main formed elements of the cervix are collagen and elastin and there are also numerous fibroblasts. Fibrils of collagen are amassed into bundles. The collagen fibril is made up of tropocollagen molecules arranged in a staggered longitudinal way to form the typical striated structure of collagens types I and III; about 70% of cervical collagen is type I and the remaining 30% is type III (Kleissl *et al* 1978, Danforth 1983, Minamoto *et al* 1987). Collagen is resistant to the majority of extracellular proteases but can be broken down by collagenase produced by fibroblasts and leukocytes and leukocyte elastase which is produced by macrophages, polymorphs and eosinophils. The collagen bundles are embedded in a matrix of ground substance comprising proteoglycan complexes. These proteoglycans contain a variety of glycosaminoglycans (GAGs). GAGs are acid mucopolysaccharides and often contain a large number of sulphate groups which give the molecule highly hydrophilic properties. There are a range of GAGs, such as heparan, dermatan and chondroitin. The predominant GAGs found in the cervix are chondroitin and dermatan sulphate (Kokenyesi 1991, Uldberg *et al* 1983, von Maillot *et al* 1979), which are important in orientating the collagen fibrils (Scott *et al* 1981). They are also important in providing mechanical strength (Golichowski 1980, Lindahl *et al* 1978). The binding affinity of GAGs rises with increasing chain length and charge density. Hyaluronic acid is quantitatively another key component of GAGs in human cervical tissue but is not sulphated and therefore binds least strongly of the GAG molecules and will act to destabilise the collagen fibrils (Greer 1992). GAGs containing iduronic acid as opposed to glucuronic such as dermatan sulphate bind strongly and promote tissue stability (Obrink 1973). Alterations in the proteoglycans/GAG arrangement can therefore alter the collagen binding and facilitates collagen breakdown.

### **1:2.2. Changes in cervical connective tissue.**

Data obtained from cervical biopsies have shown that during labour and prior to the onset of labour, profound changes occur in the organisation of cervical connective tissue. The collagen content decreases by 70% in comparison with concentrations in the non-pregnant cervix (Ekman *et al* 1991), and the concentration of GAGs increases about 2.5 fold during the same period (Rath *et al* 1991). From the start of labour until full dilatation and the immediate post-partum period there is a steep fall in cervical GAG levels mainly from a loss of dermatan sulphate, followed by a loss of hyaluronic acid and of heparan and chondroitin sulphates (Rath *et al* 1991). These alterations in the ground substance cause a structural disorganization of collagen fibrils. This change is likely to facilitate their enzymatic degradation. In the non-pregnant state, the cervix is composed of around 80% water (Liggins 1978) and this escalates close to 86% in late pregnancy (Uldbjerg *et al* 1983). As GAGs are hydrophilic these molecules may have a key role in controlling tissue hydration, with increasing hydration the collagen fibrils are destabilised and cervical ripening is advanced. The collagen fibrils and GAGs are produced by fibroblasts, which are the main orchestrator of the tissue changes in the cervix. Fibroblast activation takes place and local prostaglandin production increases. Inflammatory cells infiltrate the cervix at term in parallel with this ripening process and the cervical stroma becomes oedematous and highly vascularised (Liggins 1981).

### **1:2.3. Physiological mechanism in cervical ripening.**

The physiological mechanism underlying cervical ripening is uncertain. The alterations in collagen composition is likely to reflect changes in the proteoglycan/GAG composition of the ground substance as noted above. In addition there may be increased enzymatic

collagen degradation due to increased collagenolysis induced by fibroblasts and leukocytes. Collagenase will degrade collagen types I, II and III. Studies have shown that as the cervical collagen content decreases in pregnancy the leukocyte elastase and collagenase activity intensify (Uldberg *et al* 1983) with a dramatic rise in collagenase in the cervix in active labour (Rajabi *et al* 1988). The action of collagenase is highest during the active phase of labour (cervical dilatation 6-8cm), this is believed to reflect collagenase carried into the cervix by infiltrating neutrophils rather than increased collagenase production by cervical fibroblasts (Osmers *et al* 1992). These enzymes can also breakdown proteoglycans, and as GAGs play a role in orientating the collagen fibrils they could reduce the mechanical strength of the collagen fibrils without involving collagen degradation as such. Thus cervical ripening is due to changes in GAG composition, tissue hydration and collagen breakdown and thus appears to be mediated by fibroblast activation and infiltration of activated lymphocytes.

#### **1:2.4. Prostaglandins in cervical ripening.**

Prostaglandins have a physiological role in cervical ripening and can rapidly induce ripening pharmacologically at any stage of pregnancy (Calder *et al* 1991, Calder 1980). The main prostaglandins produced by the cervix are PGE<sub>2</sub>, PGI<sub>2</sub> and to a lesser extent PGF<sub>2α</sub> and their production increases at term (Ellwood *et al* 1980). There are basically two routes by which prostaglandins could lead to cervical ripening, first they could induce collagen breakdown, and second, they could alter collagen binding and tissue hydration by changing the GAG/proteoglycan composition (Uldbjerg *et al* 1983). Animal studies have demonstrated that PGE<sub>2</sub> causes an increase in hydration and hyaluronic acid concentration (Carbol *et al* 1987). This may be due to either an induction of fibroblast hyaluronic synthase (Murota *et al* 1977) or induction of proteolytic breakdown of proteoglycan

complexes. Hyaluronic acid increases in the human cervix at the onset of labour (Rath *et al* 1991) and has been shown to increase interleukin - 1 (IL-1) production by human peripheral monocytes and rabbit peritoneal macrophages. IL-1 production was also stimulated with keratan sulphate and heparan sulphate and with bacterial lipopolysaccharide (Ito *et al* 1988a). Studies have also shown that the addition of human recombinant interleukin - 1 (hrIL-1) to pregnant rabbit cervical fibroblasts in vitro resulted in a sharp rise in levels of collagenase and stromelysin (an endogenous procollagenase inhibitor) and a decrease in levels of tissue metalloproteinase inhibitor (Ito *et al* 1988b). These results have been corroborated in human cervical fibroblasts (Takahashi *et al* 1991), and propose that an increase in GAG levels or in bacterial products in the cervix prior to labour could act as a signal to activate local macrophages and/or infiltrating leukocytes to release IL-1 which would then trigger the collagenolytic cascade. This is in accord with the concept that cervical ripening is an inflammatory response (Liggins 1981) and this is supported by histological evidence showing marked leucocyte infiltration in cervical biopsies intrapartum (Junqueira *et al* 1980, Bokstrom *et al* 1997).

#### **1:2.5. Oestrogen and progesterone in cervical ripening.**

There are agents that also participate in the control of cervical structural changes. Oestrogens modulate the synthesis of GAGs in a variety of tissues and increase collagen turnover in guinea-pig uterus (Rajabi *et al* 1991) changes. Oestradiol can stimulate prostaglandin production where there has been earlier exposure to progesterone, and has been employed to bring about cervical ripening in the clinical situation with limited success (Allen *et al* 1989, Gordon *et al* 1977). Progesterone appears to have an inhibitory effect on cervical ripening and parturition in animals where a decrease in progesterone at term results in cervical ripening and in labour. This progesterone decrease does not occur

in humans, but progesterone is an effective anti-inflammatory agent (Sitteri *et al* 1997) and may well be a significant physiological inhibitor of cervical ripening *in vivo* by inhibiting neutrophil influx and activation (Jeffrey *et al* 1980). Anti-progestins are used as effective cervical ripening agents in women e.g. RU 486.

#### **1:2.6. Relaxin in cervical ripening.**

Relaxin is a polypeptide produced by the placenta during pregnancy. In animals it has been shown to induce cervical ripening as well as uterine contractility (Steinetz *et al* 1995, Bryant-Greenwood 1991). In humans it has been shown to have some effect on cervical ripening (McKenzie 1993, McLennan *et al* 1980) but when recombinant relaxin was administered vaginally it was not associated with clinically effective cervical ripening (Brennan *et al* 1997). Relaxin may have some role in the ripening process but the understanding of the mechanism is unclear. Relaxin can increase the secretion of collagenase from human chorio-amniotic tissue and interfere with collagen and collagenase production from fibroblasts (Unemori *et al* 1992, MacLennan *et al* 1986). Human fibroblasts exhibit relaxin receptors and relaxin has a mitogenic effect on fibroblasts (McMurty *et al* 1980).

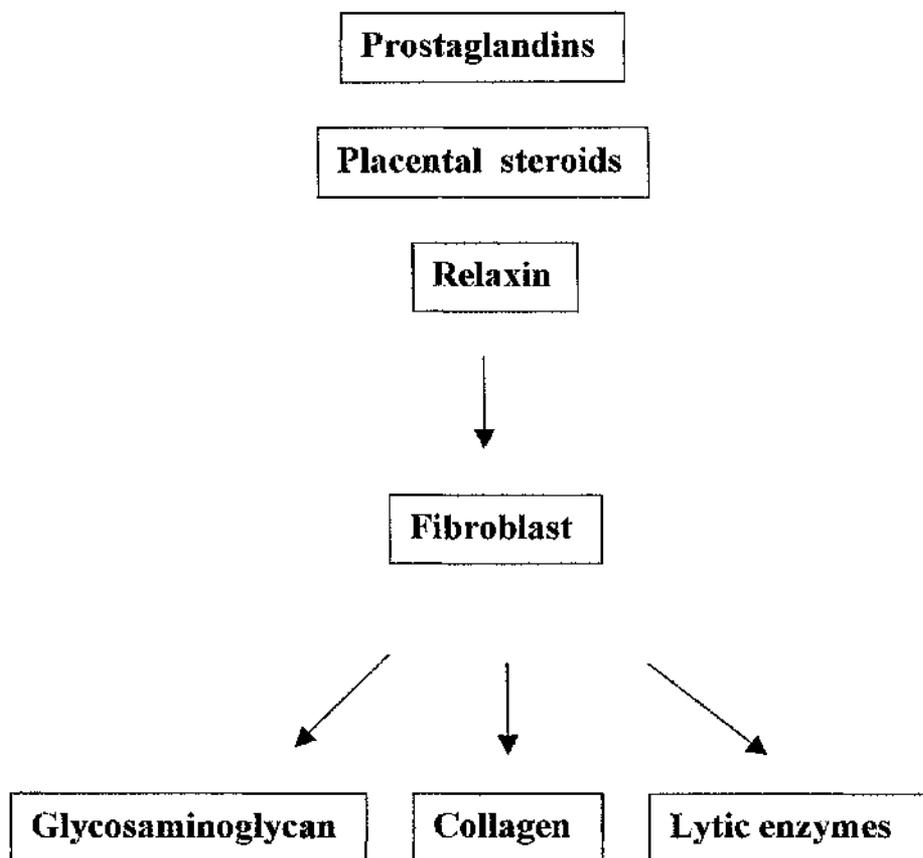
#### **1:2.7. Nitric oxide in cervical ripening.**

Nitric oxide has been proposed as a potential physiological ripener of the cervix. Animal studies have demonstrated that nitric oxide synthase, mainly the inducible form of nitric oxide synthase (Ledingham *et al* 2000, Buhimschi *et al* 1996) and nitric oxide production are involved in the physiology of cervical ripening (Garfield *et al* 1998, Chwalisz *et al* 1997). From a pharmacological perspective it has also been demonstrated that the NO-donor isosorbide mononitrate administered vaginally in early human pregnancy is capable

of inducing cervical ripening prior to termination of pregnancy (Ledingham *et al* 2001, Thomson *et al* 1997, 1998).

While several key mediators of cervical ripening have been identified further research is required to establish which of these factors precede and cause cervical ripening and how they interact. **Figure 1d.** below illustrates a summary of the agents involved in the ripening process.

**Figure 1d.** Mediators in cervical ripening.



### **1:3. Myometrium in labour.**

#### **1:3.1. Myometrial structure in labour.**

The myometrium is the outer muscular part of the uterus. It consists of bundles of nonstriated fibres, intermixed with connective tissue, nerves, blood and lymph vessels. The myometrium is formed from an outer longitudinal muscle layer running parallel to the long axis of the uterus and an inner circular muscle layer, perpendicular to the long axis. There is a marked increase in the coordination between the circular and the longitudinal myometrial muscle during labour, at which time the longitudinal muscle appears to pace the circular tissue (Tomiyasu *et al* 1988). During pregnancy oestrogens stimulate an increase in myometrial bulk, primarily by increasing muscle cell size from about 50-500µm (hypertrophy) and there is also a build up of fibrous connective tissue most of which is collagen, together with an increase in elastin content (Cunningham *et al* 1997). Functionally this arrangement of muscle cells acts as a syncitium, the cells being joined electrically via gap junctions. This allows the co-ordination of the spread of current and thereby contraction through the myometrium. Studies have shown that myometrial gap junctions are present in large numbers during term and preterm labour (Garfield *et al* 1981, 1978). These intercellular (or gap) junctions are formed as a result of interactions between intramembranous proteins within adjoining cell membranes. The proteins forming gap junctions are called connexins and are categorized by their molecular weights (Lowenstein 1981, 1987). Cx-43 has been identified as the subunit protein of the gap junction. The expression of Cx-43 in ovine myometrium increases with the onset and progression of ACTH-induced labour (Lye 1994). The complex development of gap junctions is poorly understood but in the myometrium oestrogen and progesterone are

thought to have a role in a number of the actions (Lye *et al* 1993, Petrocelli *et al* 1993, Garfield 1988). There is limited support to suggest the presence of cell adhesion molecules is necessary for gap junction formation (Mege *et al* 1988). The relative quiescence of the myometrium during pregnancy and in addition the high level of contractile activity during labour are controlled by a multifarious interaction of steroids, peptides and prostaglandins. These hormones probably exert endocrine, paracrine and autocrine effects, these effects superimposing on the earlier existing myogenic activity of both the circular and longitudinal muscle layers.

### **1:3.2. Activation of the myometrium.**

Three primary hypotheses have been proposed to explain how the uterus moves from the relatively quiescent state of pregnancy to the highly active state in labour. They are:

- it is suggested that various inhibitors, including progesterone, maintain the quiescent state of pregnancy and their removal allows the myometrium to enter the contractile state of labour.
- uterine sensitisation takes place late in pregnancy, and occurs in response to uterotrophins including oestrogen, preparing the uterus to carry out the contractions typical of labour.
- it is suggested that once sensitised, the uterus is stimulated to contract by uterotonins such as oxytocin and the stimulatory prostaglandins PGE<sub>2</sub> and PGF<sub>2α</sub>.

### **1:3.3. Myometrial quiescence.**

The relative quiescence of the uterus throughout gestation has been attributed to progesterone (Csapo 1956). In many species, progesterone-dominated uterine muscle is noncompliant to the stimulatory effects of oxytocin and prostaglandins (Csapo 1997).

Administration of exogenous progesterone to several species will decrease uterine activity (Lye *et al* 1978) and delay parturition (Pertocelli *et al* 1993). Progesterone could achieve these effects through several mechanisms, including the uncoupling of excitation-contraction, inhibition of the synthesis of agonist receptors, inhibition of gap junction formation and the control of the production or formation of prostaglandins and inhibition of pituitary oxytocin release. The major effect of progesterone is to reduce myometrial responsiveness and conductivity but not to block the spontaneous contractility of the myometrial cells.

#### **1:3.4. The physiology of myometrial contractility.**

The contraction of myometrial cells is dependent on an increase in intracellular calcium ion concentration, both by release from intracellular stores and by influx into the muscle cells from the extracellular fluid. The calcium then attaches to regulatory sites on the contractile proteins actin and myosin. Myosin is both a structural protein and an enzyme capable of converting the chemical energy of ATP into the mechanical energy of muscle contraction. The myosin molecule has two functional components: the head, where actin-myosin interaction occurs and where the ATPase sites are located; and the tail, which plays a part in the formation of myosin filaments. The actin and myosin filaments slide past each other during contraction, and the myosin heads and actin molecules develop cross-bridges that cause the contractile force of labour. The calcium concentration is of basic importance in the regulation of actin-myosin interaction but the precise mechanism is not fully understood. An acknowledged theory is that calcium in combination with calmodulin, activates the enzyme myosin light-chain kinase (MLCK) which phosphorylates a 20-kDA light chain of myosin (McKenzie *et al* 1990). Phosphorylated myosin interacts with actin causing contractions and when calcium is removed

dephosphorylation occurs rapidly and the muscle relaxes. To sum up,  $\text{Ca}^{2+}$  activates MLCK which phosphorylates the 20-kDA myosin light chains thus increasing ATPase activity and the rate of cross-bridge formation.

### **1:3.5. Oestrogen in the myometrium.**

The majority if not all of the actions of uterine activation are considered to be as a consequence of the action of oestrogen. The uterine activities of oestrogen are in many respects antagonistic to those of progesterone. Administration of oestradiol ( $\text{E}_2$ ) to the pregnant ewe can cause an elevated oxytocin response (Liggins *et al* 1977) and lead to premature labour (Cahill *et al* 1976). In women the function of oestrogen in the control of myometrial contractility is poorly understood. The maternal plasma concentration of oestrogen is very high at term but much of this is conjugated (Yen 1984). Oestrogens do stimulate growth and actomyosin formation in the uterus, additionally they are most likely to be crucial for the maintenance of an effective resting membrane potential to facilitate production of action potentials (Finn *et al* 1975). Oestrogen is able to act at several levels to increase the ability of the myometrium to contract and respond to agonists. Taking into consideration the varied effects of this steroid in other species it would be unexpected if it was not of key significance in the human.

### **1:3.6. Prostaglandins in the myometrium**

Prostaglandins ( $\text{PGE}$ ,  $\text{PGE}_{2a}$ ) are powerful and rapid stimulants of myometrium in the majority of animal species, functioning through receptor linked mechanisms (Goureau *et al* 1992, Molnar *et al* 1990, Wikland *et al* 1984). The rise in spontaneous activity in the human is linked to an increase in the ratio of stimulatory ( $\text{PGE}$ ,  $\text{PGF}$ ) to inhibitory ( $\text{PGI}_2$ ) prostaglandins, which could be directed by oestrogen (Olsen *et al* 1983). In studies using a

sheep model in which preterm labour was induced, an increase in the production of stimulatory prostaglandins in intrauterine tissues and peripheral plasma prior to the onset of uterine contractile activity (Olsen *et al* 1984) was demonstrated. In addition labour can be blocked by administration of inhibitors of prostaglandin synthesis in many species including human (Amy *et al* 1984).

### **1:3.7. Oxytocin in the myometrium.**

Oxytocin (OT) can stimulate myometrial contractions in all species depending upon the steroid environment. Plasma oxytocin levels do not increase at term or in the first stage of labour, but around the onset of labour in the uterus there is a marked sensitivity to oxytocin. This is associated with both an upregulation of OT receptor mRNA levels and an increase in the number of myometrial oxytocin receptors (Fuchs *et al* 1995, Kimura *et al* 1992), which may enable the myometrium to react to circulating levels of oxytocin that are ineffective in the nonpregnant state. Oxytocin can increase endometrial prostaglandin production (Roberts *et al* 1976) and it can also stimulate myometrial activity directly (Windmoller *et al* 1983). The mechanism of action of prostaglandin and oxytocin are not fully understood. Both act to increase intracellular calcium levels (Carsten *et al* 1977, Soloff *et al* 1972) and prostaglandins are understood to be involved in the aggregation of gap junction proteins, while oxytocin and prostaglandins may act to control these channels (Garfield 1984).

### **1:3.8. Summary.**

In summary the quiescence of the myometrium during pregnancy and the intensity of contractile activity during labour are controlled by a complex interaction of steroids, peptides and prostaglandins. The steroids (oestrogen and progesterone) most likely modify

the activity of the myometrium through mechanisms related to changes in the synthesis of inhibitory and stimulatory regulators and their receptors. Prostaglandins and peptides effect direct and indirect stimulation or inhibition of the myometrium through direct activation or inactivation of proteins such as myosin light chain kinase, calmodulin etc, and indirectly through proinflammatory changes. Therefore during pregnancy the quiescent and relatively unresponsive myometrium is maintained by the collective effects of progesterone, PGI<sub>2</sub> and relaxin. At term the relative oestrogen dominance allows the myometrium to become responsive to prostaglandin (PGF) and oxytocin which can then activate the regulatory and contractile proteins.

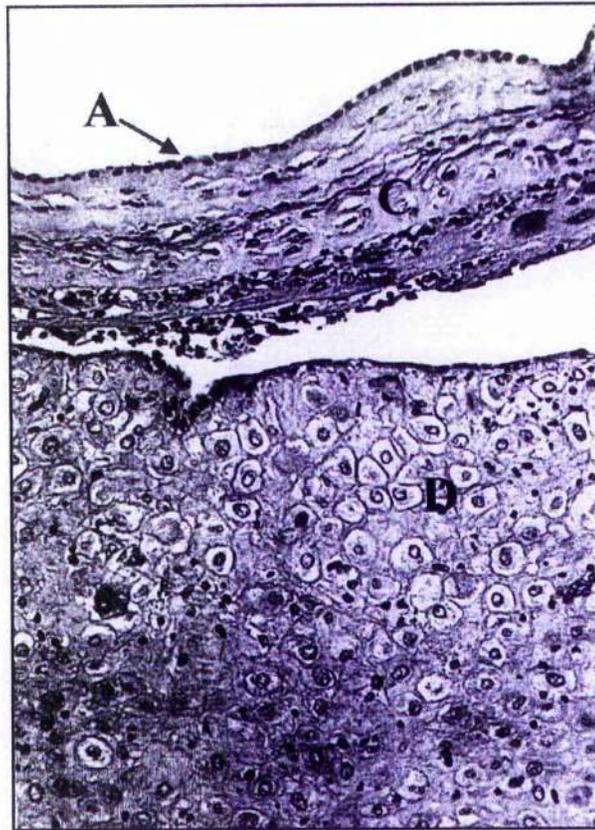
#### **1:4. Biochemistry of the Fetal Membranes.**

The human fetal membranes are genetically the same as the fetus and form a highly specialised interface between mother and fetus pivotal to the maintenance of pregnancy and parturition at term. The fetal membranes comprise of three distinct layers. The inner layer, the amnion is a thin avascular layer of epithelial cells and connective tissue. The amnion is passively connected to the next layer the chorion laeve, by the internal pressures of the amniotic fluid. The composition of the chorion laeve is more complex. Within the connective tissue there are small fetal (chorioallantoic) blood vessels and on the outer surface of the chorion extravillous cytotrophoblasts are found. The cytotrophoblasts intermingie with the outermost layer of the fetal membranes the decidua capsularis. The decidua is the only maternal constituent of the membranes. A variety of cells are present within the decidua which include trophoblasts, phagocytes and other inflammatory cells such as large granular lymphocytes. Maternal blood vessels are also contained within the decidua.

The illustration below of the fetal membranes (**fig 1e**) based on *Anatomy and Pathology of the Placental Membranes*. In: *Pathology of the Human Placenta* eds: Kurt Benirschke and Peter Kaufmann.

**Figure 1e.**

The three layers of the fetal membranes (A) the amnion,(C) the chorion,(D) the decidua.



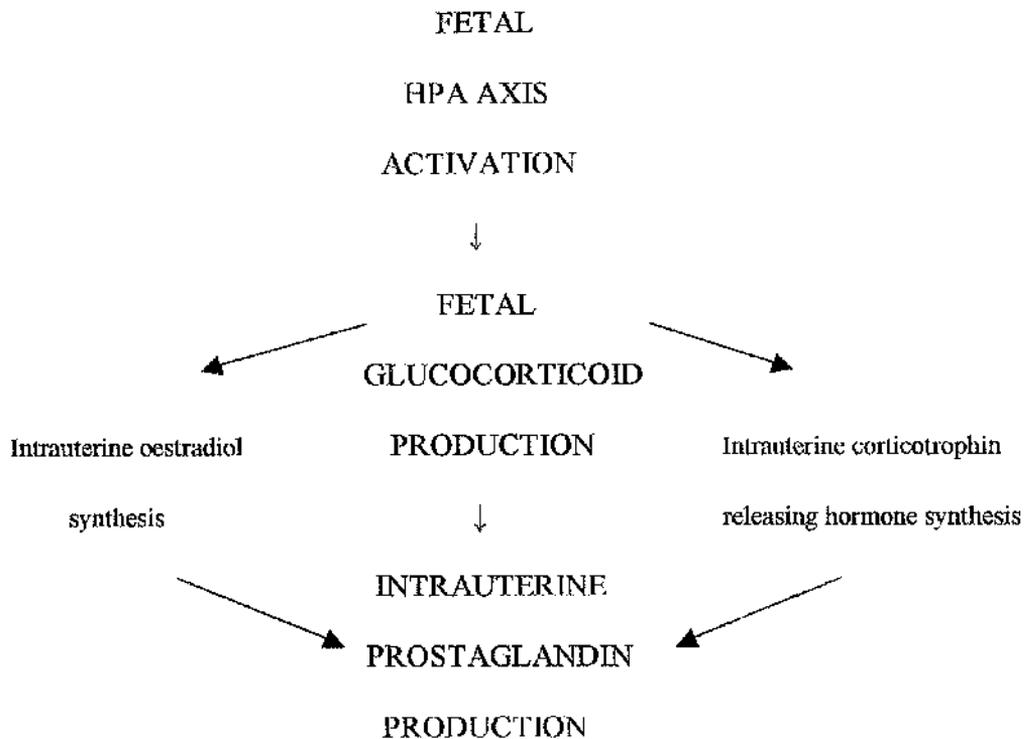
Most information comes from ovine parturition but it may be compared with human parturition. Parturition in both ovine and human is associated with activation of hypothalamic-pituitary-adrenal (HPA) function in the fetus and the enhanced effect of glucocorticoids on trophoblasts cells of the placenta and fetal membranes (Challis 1997).

Early studies in sheep have shown that maturation of the fetal HPA axis, which effects an increase in fetal adrenal cortisol secretion, is important for the stimulus of the onset of labour (Liggins *et al* 1973). In this species, maturation of the HPA axis over the final 30-35 days of pregnancy is likely to be due to an upsurge in corticotrophin releasing hormone (CRH) production from the fetal hypothalamus, which stimulates pituitary adrenocorticotrophic hormone (ACTH) release and which in turn stimulates adrenal cortisol production (Challis *et al* 1989). In many mammalian species studied near the end of gestation and at the onset of labour an increase in fetal plasma glucocorticoid concentration has been demonstrated due to maturation and the continued activation of the HPA axis (Fowden *et al* 1998). These glucocorticoids induce maturational transformations in the fetal organs such as the lungs, kidneys, liver and gut in readiness for delivery. This surge in glucocorticoid has been proposed to be essential to the cascade of actions leading to the onset of parturition (Challis *et al* 1997). In ovine intrauterine tissues this increase in cortisol is considered to be responsible for the decrease in progesterone production by the increase in the synthesis of oestrogen and the increase of intrauterine prostaglandin production through the induction of prostaglandin H synthase type II (PGHS-II) (Challis *et al* 1997). Oestradiol stimulates the expression of specific contraction associated proteins (CAPS) within the myometrium allowing it to contract in response to uterotonins such as prostaglandin and oxytocin (Lye 1994) resulting in myometrial contractions, labour and delivery of the fetus. In the human the elevated fetal adrenal glucocorticoid levels effect prostaglandin production via the glucocorticoid-stimulated intrauterine corticotrophin releasing hormone (CRH) (Whittle *et al* 2001). These events are summarised in **Figure 1f** below.

**Figure 1f.** HPA / axis in ovine and human parturition.

**Ovine Parturition**

**Human Parturition**



Based on Whittle et al. in *Biology of Reproduction* 64:2001.

Prostaglandin production within human fetal membranes is regulated by the various layers of the membranes (Challis *et al* 1994). Concentrations of prostaglandins in amniotic fluid could give an estimated indication of prostaglandin synthesis within intrauterine tissues, mainly in the amnion and chorion but they may not reflect the concentrations of prostaglandins at the critical choriodecidua or myometrial interface. Most of the PGE<sub>2</sub> in amniotic fluid is likely to be produced by the amnion. Studies have shown that production of PGE<sub>2</sub> from amnion tissue obtained from women in spontaneous labour is significantly

higher than that obtained from women at elective caesarian section prior to the onset of labour (Skinner *et al* 1985). The chorion also produces PGE<sub>2</sub> and prostaglandin dehydrogenase (PGDH) enzyme by which it metabolises and averts the access of PGE<sub>2</sub> produced within the amnion or chorion from reaching the underlying decidua (Nakla *et al* 1986). Therefore prostaglandins produced within the decidua, or within the myometrium itself may have a key role in supplying the stimulus to myometrial contractility at term. The role of prostaglandins produced within the amnion and chorion is poorly understood but these PGs may have a similar function to those produced in the cervix which are associated with the disarrangement of the collagen bundles which in the fetal membranes could lead to their weakening and rupture.

#### **1:5. Labour as an inflammatory response.**

Parturition has been likened to an inflammatory process (Steinborn *et al* 1999, Thomson *et al* 1999, Liggins 1991). The main features of an inflammatory response are vasodilation (widening of the blood vessels to increase the blood flow to the affected area), increased vascular permeability which allows diffusible components to enter the appropriate site and cellular infiltration by chemotaxis which is the directed movement of inflammatory cells through the walls of the blood vessels into the required site. The early events of inflammation are brought about by the release of cytokines and later vascular events are mediated by products of arachidonic acid. These events can be brought about by inflammatory mediators in particular the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF  $\alpha$ . The release of the cytokines IL-1 $\beta$ , IL-6 and TNF  $\alpha$  is responsible for the very early events that occur in inflammation, IL-8 is known to be a potent chemokine. In the initial stage of the inflammatory process these cytokines are secreted by local tissue macrophages in response to various mediators e.g. in infection through bacterially derived

lipopolysaccharides (LPS). These early response cytokines are capable of inducing the expression of cell adhesion molecules on the surface of vascular endothelial cells (Dustin *et al* 1986). These adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leucocyte adhesion molecule-1 (ELAM-1), are involved in the attachment of leucocytes to the vascular endothelium and their extravasation from the circulation into the affected tissues (Bevilacqua 1993, Akyama *et al* 1989, Wawryk *et al* 1989). The ensuing inflammatory cascade causes the production of arachidonic acid metabolites, which along with other inflammatory effects leads to cervical ripening, uterine contractions and subsequent delivery (Edwin *et al* 1993, Lundin-Shiller *et al* 1991). Studies in the aetiology of labour have demonstrated that an increase in the intra-uterine concentrations of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF $\alpha$  are a main cause of infection-associated uncontrollable preterm labour and subsequent premature delivery (Cherouny *et al* 1993, Romero *et al* 1992a, Romero *et al* 1992b, Santhaman *et al* 1991). These cytokines are considered to have a key role in the intra-uterine response to infection. Cytokines, particularly IL-1, TNF $\alpha$  and IL-6, are crucial in driving the acute phase response. There is evidence that inflammatory processes linked with raised cytokine production are also present in normal term labour as well as preterm labour which is not due to intra-amniotic infection. Elevated levels of IL-1 $\beta$ , IL-6 and TNF  $\alpha$  were detected in both amniotic fluid and cervical secretions during spontaneous term labour (Steinborn *et al* 1996, Opsjon *et al* 1993). Studies have also shown increased levels of IL-1, IL-6 and IL-8 in choriodecidual and placental tissue in association with term and pre-term labour (Laham *et al* 1999, Keelan *et al* 1999, 1997). Choriodecidual tissue in culture has been shown to produce IL-6 and TNF  $\alpha$  (Dudley *et al* 1992, Kameda *et al* 1990, Casey *et al* 1989). The potent chemokine IL-8 has been demonstrated in human cervical tissue

(Barclay *et al* 1993) and in choriodecidual tissue (Kelly *et al* 1992). Levels of IL-8 increase in parallel with increasing cervical dilatation in tissue from the lower segment in term pregnant women (Winkler *et al* 1999a, 1998, Osmer *et al* 1995). Elevated levels of IL-8 have been noted in amniotic fluid in pre-term and term pregnancies (Cherouny *et al* 1993, Laham *et al* 1993, Saito *et al* 1993, Romero *et al* 1991). Inflammatory cells are known to infiltrate uterine tissue. The inflammatory cells within the ripened cervix are predominantly neutrophils and macrophages which accumulate in the stroma before the onset of labour at term (Bokstrom *et al* 1997). These leukocytes contribute to cervical remodelling by releasing proteolytic enzymes such as collagenase and elastase (Osmer *et al* 1992, Rajabi *et al* 1988). Elsewhere in the uterine tissues inflammatory cells again principally macrophages and neutrophils, have been shown to infiltrate the placenta, maternal decidua and the fetal membranes during parturition and may play a role in spontaneous rupture of membranes (Rosenberg *et al* 1996, Halgunset *et al* 1994). It has been demonstrated that leukocytes infiltrate both the upper and lower myometrium at term (Thomson *et al* 1999). The involvement of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF  $\alpha$  in uterine tissue during parturition and the infiltration of inflammatory cells in uterine tissue before and after the onset of labour are consistent with parturition being an inflammatory process.

## **1:6. Cytokines in parturition**

### **1:6.1. The function of cytokines known to be associated with human parturition.**

Cytokines are simple polypeptides or glycoproteins with a molecular weight  $\leq$  30kD. They are produced as part of the acute phase response by a variety of stimuli. They are secreted by most nucleated cell types in the body and their pleiotropic actions include numerous

effects on cells of the immune system and modulation of inflammatory responses (Vilcek 1991). Cytokines are produced in the golgi of the cell and can travel through the endoplasmic reticulum to be released as soluble mediators, they are possibly processed to cytosolic forms that can transfer intracellularly or continue attached to the membrane. Cytokines consequently mediate autocrine function through release, membrane expression or intracellularly within the cell. Cytokines can also function in a paracrine mode, permitting cellular communication further than that made possible by cell-cell contact. Cytokines trigger numerous intracellular signalling pathways to facilitate their physiological effects. The receptors for several pro- and anti-inflammatory cytokines bind to members of the JAK (Janus kinases) family of tyrosine kinases and activate downstream STAT (signal transducer and activation of transcription) signalling (Imada & Leonard 2002). SOCS (suppressors of cytokine signalling) proteins function as intracellular regulators of cytokine signalling by attaching to members of the JAK family, consequently inhibiting kinase activity as well as subsequent phosphorylation and activation of downstream targets. Today cytokine nomenclature is based on the term interleukin proposed in 1979 by the participants at the Second International Lymphokine Workshop. The term interleukin (IL) derives from the ability of these proteins to act as communicative signals between different populations of leukocytes. Whereas many cytokines are termed interleukins others continue to be known by their older names, e.g. tumour necrosis factor  $\alpha$  (TNF $\alpha$ ).

### **1:6.2. Interleukin-1 (IL-1).**

Interleukin-1 (IL-1) exists in two isoforms termed IL-1 $\alpha$  and IL-1 $\beta$  which are homologous but genetically distinct. IL-1 is secreted as a 17-kDa peptide following proteolytic cleavage from a 31-kDa precursor (Dinarello1991a). Both forms are synthesised primarily

by cells of the monocyte-macrophage lineage in response to various inducers including endotoxin and TNF  $\alpha$ . IL-1 is a powerful pro-inflammatory cytokine mediating various biological and pathophysiological activities which can be either localised to the site of IL-1 release, so called local effects or systemic. The many actions brought about by IL-1 include, stimulation of arachidonic acid metabolism; elevation of basal body temperature; neutrophilia; upsurge of acute phase reactants; enhancement of the production of extracellular matrix components e.g. collagen type IV; increasing expression of cell adhesion molecules e.g. ICAM-1, ELAM-1 and VCAM-1; rising antibody production; increasing cytokine expression; increasing expression of cell surface IL-2 receptors on T-lymphocytes; and enhancement of cell proliferation e.g. fibroblasts, vascular smooth muscle cells, renal mesangial cells. The effects of IL-1 are applied via binding specific cell surface receptors. Both forms of IL-1 bind to the same receptor and consequently show similar if not identical biological activities too. The IL-1  $\beta$  but not the IL-1 $\alpha$  precursor must be processed before it can bind to the receptor. Two types of receptor of IL-1 that bind with different affinities have been described. IL-1 $\alpha$  and IL-1 $\beta$  block the attachment of each other to the receptor. The receptor isolated from T-cells is expressed predominately on T-cells and cells of mesenchymal origin and is called Type-1 receptor. Type-1 receptor binds both types of IL-1 with equal affinity. The Type-2 receptor is isolated from B-cells, granulocytes and macrophages. In addition both types of IL-1 receptors bind the IL-1 receptor antagonist IL-1ra. Inflammatory cytokines such as IL-1 have been implicated as paracrine mediators of gestational tissue prostaglandin production and extracellular matrix remodelling, these are two vital functions in the progression of parturition.

### **1:6.3. Interleukin-6 (IL-6).**

Interleukin-6 (IL-6) is a 26-kDa multifunctional cytokine implicated as being a key mediator of the host defence response to infection and tissue damage, regulation of immune responses, acute phase reactions and haematopoiesis (Hirano 1991). IL-6 expression is induced by numerous inflammation-associated cytokines including IL-1, TNF  $\alpha$ , IL-2, interferon  $\beta$ , lipopolysaccharide and several viruses. It is produced by a diverse number of cells such as fibroblasts, monocyte/macrophages, endothelial cells, keratinocytes and endometrial stromal cells. Elevation of intracellular cAMP concentration by protein kinase C, calcium ionophores and a range of other agents also induce IL-6 biosynthesis. IL-6 acts on the hypothalamo-pituitary axis to increase cortisol which in turn inhibits IL-6 gene expression (Hirano 1991). Additionally, IL-6 inhibits the production of IL-1 and TNF, both of which are powerful inducers of IL-6. IL-6 brings about important transformations in the biochemical, physiological and immunological status of the host including the acute phase protein response, activation of T and natural killer cells and stimulation and proliferation of immunoglobulin production by B cells (Dinarello 1991). The IL-6 receptor has been designated CD 126, it is expressed on T-cells, mitogen activated B-cells, peripheral monocytes and some macrophage- and B-cell – derived tumour cell types. In numerous cell types the IL-6 receptor expression is enhanced by glucocorticoids. It has been hypothesized that IL-6 production is an element of a generalised mechanism of decidual activation in preparation for parturition (McDonald *et al* 1991).

### **1:6.4. Interleukin-8 (IL-8).**

Interleukin-8 (IL-8) is a non-glycosated protein of 8kDa (72 amino acids). It is one of several inflammatory cytokines that possess chemotactic activity and are referred to as

chemokines (Baggioni 1989). IL-8 differs from other chemokines in its ability to specifically activate neutrophil granulocytes hence it is also known as neutrophil attractant/activating protein (NAP-1). In neutrophils IL-8 causes a short-lived rise in cytosolic calcium levels and the release of enzymes from granules. IL-8 also increases the metabolism of reactive oxygen species, enhances chemotaxis, increases expression of cell adhesion molecules and promotes the adherence of neutrophils to endothelial cells (Braun 1993). IL-8 is produced by stimulated monocytes but not tissue macrophages and T-lymphocytes. IL-8 is also produced by macrophages, fibroblasts, endothelial cells, keratinocytes, melanocytes, hepatocytes, chondrocytes and a number of tumour cell lines. The IL-8 receptor termed CD128 is expressed in various cell types including those not responding to IL-8. The IL-8 receptor is a member of a G-protein-coupled receptor family (GPR). There are at least two different IL-8 receptor types. Type-1 receptor specifically binds IL-8, the type-2 receptor also binds the IL-8 related factors MGSA (Melanoma growth stimulatory activity), MIP-2 (macrophage inflammatory protein), and NAP-2 (neutrophil-activating protein-2). In gestational tissues, a key role of IL-8 is as a chemattractant and activator of neutrophils (Maehara *et al* 1996).

#### **1:6.5. Tumour Necrosis Factor Alpha (TNF $\alpha$ ).**

TNF  $\alpha$  is a non-glycosated protein of 17 kDa (157 amino acids). Although structurally distinct from IL-1 it possesses similar biological activities (Dinarello 1991b, Aggarwal *et al* 1985). TNF  $\alpha$  has a broad spectrum of biological activities including inducing the expression of genes, effecting mechanisms of signal transduction and initiating apoptosis. TNF  $\alpha$  is a pleiotropic cytokine involved in numerous diseases associated with inflammation, cachexia, shock and tissue injury. Macrophages and other cells produce TNF  $\alpha$  in different infectious and non-infectious diseases (Manogue 1991). In resting

macrophages TNF  $\alpha$  induces the synthesis of IL-1 and prostaglandin E<sub>2</sub>. In the body TNF  $\alpha$  and other cytokines are involved in restoring normal homeostasis when endangered by inflammatory mediators but overproduction of cytokines could in fact have damaging effects to the host. The TNF $\alpha$  peptide is recognized by two separate receptors TNFR1 or p55 and TNFR2 or p75. p55 is predominantly expressed on cells receptive to the cytotoxic action of TNF. p75 is present on various cell types of myeloid origin, it is expressed strongly on stimulated T-cells and B-lymphocytes. Differential effects of the two receptor subtypes are present in TNF-mediated adhesion of leucocytes to the endothelium. It seems the engagement of the p55 receptor specifically leads to the induction of the cell adhesion molecules ICAM-1, E-selectin, V-CAM and CD44, while the engagement of both p55 and p75 receptor induces the expression of Alpha-2 integrin. Like IL-1, TNF $\alpha$  induces the formation of PG and expression of extracellular remodelling enzymes in gestational tissues.

**1:7.1. Hypothesis**

Labour is an inflammatory process characterized by an influx of leukocytes in uterine tissue with an upregulation of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF $\alpha$  in cervix, myometrium and fetal membranes during labour.

**1:7.2. Aims**

The aims of this thesis were to localize the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF $\alpha$  using immunocytochemistry in uterine tissue in labour and before the onset of labour. To measure the expression of IL-1 $\beta$ , IL-6, IL-8 and TNF $\alpha$  mRNA in cervix, myometrium, amnion and chorio/decidua in labour and not in labour employing northern analysis. To determine the number of leukocytes and leukocyte sub-sets including neutrophils, macrophages, T-cells and B-cells in amnion, chorion, decidua, cervix and myometrium collected during labour and before the onset of labour.

## **Chapter 2.**

### **Material and Methods.**

### **2:1. Subjects.**

The following groups of women were recruited to the study (i) pregnant women at term ( $\geq$  37 weeks gestation) undergoing elective caesarean section prior to onset of labour and (ii) pregnant women at term undergoing emergency caesarean section during spontaneous labour (cervical dilatation  $>4\text{cm}$  and  $<9\text{ cm}$ ) see **table 2a**. Women were excluded from the study if they had a multiple pregnancy, evidence of active infection or if the labour had been induced. No woman had been given glucocorticoids or steroids within 48hrs before delivery. Ideally all samples should be matched for age, gestation and parity but this was ethically and practically difficult. It is accepted that the underlying physiological processes in labour are the same no matter the age, gestation or parity. The study was approved by the Research Ethics Committee at North Glasgow Hospitals University NHS Trust. Informed consent was obtained from each woman prior to recruitment.

### **2:2. Biopsies**

Each patient had biopsies taken either from the myometrium, the cervix or the fetal membranes (see **table 2a**). Lower segment myometrial biopsies were obtained from the upper margin of the lower uterine segment incision. In twelve of the subjects additional biopsies were obtained from the upper uterine segment by dissecting a strip of myometrium from the inner aspect of the posterior uterine wall. In all myometrial biopsies, myometrium was separated from surrounding structures i.e. endometrium and decidua, by sharp dissection. Cervical biopsies from women not in labour were obtained per vaginum from the anterior lip of the cervix prior to caesarean section. Biopsies from women in labour were obtained from the same site in the cervix after delivery of the infant, the cervix being approached via the uterine incision ( $n = 8$  prior to the onset of

labour, n = 8 in labour). Biopsies of fetal membranes were full thickness and included attached decidua (n = 10 prior to the onset of labour, n = 10 in labour). All tissue specimens were divided in two, one half was fixed in 10% buffered formalin (BDH, Poole, UK) for 24 h, 50% ethanol for 8h, 70% ethanol for up to 24h. The tissue was then cut and placed in uni-cassettes. The cassette was then placed in a Histokinette 2000 and was taken through the following solutions: methylated spirits for 1h, methylated spirit phenol for 1h 30min, industrial alcohol for 1h 30min, 2h, 2h and 2h 20min, industrial alcohol/chloroform (50/50) for 1h, chloroform for 1h 30min, 2h and 2h, and 2 wax treatments for 2h and 3h 30min. The uni-cassettes were then transferred to metal uni-cassettes and embedded using aTissue Tek 3. The paraffin blocks were subsequently used in immunocytochemistry. The other half of the tissue was flash frozen in liquid nitrogen for total RNA extraction and stored at  $-70^{\circ}\text{C}$  in pre-cooled metal pots until processed. Fetal membranes were separated into amnion and choriodecidua before being flash frozen or were fixed intact in 10% buffered formalin. Only eight of the cervix biopsies had sufficient material to be split in two. Sections  $5\mu\text{m}$  thick were cut from the paraffin embedded tissues using a microtome (Leica RM 2135) mounted on silane (2% 3-aminopropyltriethoxysilane [Sigma] in acetone [BDH]) coated slides, heated to  $60^{\circ}\text{C}$  overnight and stored in slide boxes until used.

Table 2a. Patient sample list.

Patient no.	Gestation	In labour	Not in labour	Myomet LSM	Myomet USM	Amnion	Chori/dec	Cervix
120	Term		√	√		√	√	
121	Term		√	√		√	√	
122	Term		√	√		√	√	
127	Term		√	√		√	√	
128	Term	√				√	√	
130	Term		√	√		√	√	
131	Term		√	√		√	√	
132	Term	√		√		√	√	
133	Term	√				√	√	
136	Term		√	√		√	√	
137	Term		√	√		√	√	
138	Term		√	√		√	√	
139	Term		√	√		√	√	
140	Term		√			√	√	
149	Term	√		√		√	√	
154	Term	√		√				
156	Term	√		√		√	√	
160	Term	√		√		√	√	
166	Term	√		√		√	√	
168	Term	√		√		√	√	
170	Term	√		√		√	√	
171	Term	√		√		√	√	
174	Term	√		√				
AT1	Term <sup>+</sup>	√		√	√			
AT3	38		√	√	√			
AT6	39 <sup>+</sup>	√		√	√			
AT9	Term	√		√	√			
AT10	39	√		√	√			
AT11	39		√	√	√			
AT12	Term	√		√	√			
AT15	39		√	√	√			
AT16	39		√	√	√			
AT17	38		√	√	√			

Table 2a (cont).

Patient no.	Gestation	In labour	Not in labour	Myomet LSM	Myomet USM	Amnion	Chori/dec	Cervix
AT20	39		√	√	√			
14/12/98	Term <sup>+</sup>	√		√	√			
MC2	Term <sup>+</sup>	√				√	√	
MC3	Term	√				√	√	
MC4	Term	√				√	√	
MC5	Term <sup>+</sup>	√					√	
MC6	Term <sup>+</sup>	√					√	
MC7	Term <sup>+</sup>	√				√		
MC8	Term <sup>+</sup>	√				√		
MC9	Term <sup>+</sup>	√				√		
MC10	NK		√			√		
MC11	NK		√			√		
MC12	38 <sup>+</sup> wks		√			√	√	
MC13	NK		√			√	√	
MC14	38wks		√			√	√	
MC15	38wks		√			√	√	
MC18	37wks		√				√	
MC19	NK		√				√	
01EM	Term <sup>+</sup>	√						√
02EM	37 <sup>+</sup> wks	√						√
03EM	Term <sup>+</sup>	√						√
04EM	37 <sup>+</sup> wks	√						√
05EM	37wks	√						√
07EM	Term <sup>+</sup>	√						√
14EM	Term <sup>+</sup>	√						√

Table 2a (cont)

Patient no.	Gestation	In labour	Not in labour	Myomet LSM	Myomet USM	Amnion	Chori/dec	Cervix
21EM	Term <sup>+</sup>	√						√
Cx 24/7	NK	√						√
6EL	NK		√					√
11EL	NK		√					√
12EL	39wks		√					√
14EL	39 <sup>+</sup> wks		√					√
15EL	NK		√					√
16EL	38 <sup>+</sup> wks		√					√
17EL	39 <sup>+</sup> wks		√					√
18EL	38 <sup>+</sup> wks		√					√
20EL	39wks		√					√

**Myomet** - myometrium

**Chori/dec** - choriodecidua

**LSM** - lower smooth muscle

**USM** - upper smooth muscle

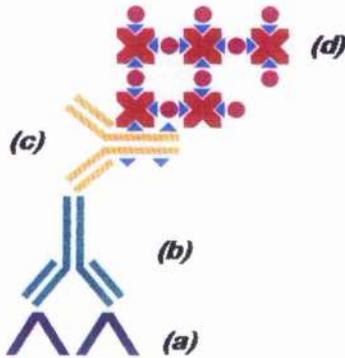
**NK** - not known

**+ - over**

### 2:3. Immunocytochemistry.

Immunocytochemistry is the application of immunological methods to cells or tissue sections to enable protein visualization by the demonstration of a marker conjugated to the final reactant. The method employed was the avidin-biotin complex (ABC) diagrammatically shown in **Figure 2a**, where **(a)** is the tissue antigen **(b)** the primary antibody **(c)** biotinylated secondary antibody **(d)** avidin biotin peroxidase complex.

**Figure 2a.**



Immunocytochemistry was performed on paraffin embedded myometrial, cervical and fetal membrane biopsies using antibodies against the cytokines IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and the white cell markers CD45 (common leucocyte antigen), CD 68 (macrophages), CD3 (T-lymphocytes), CD20 (B-lymphocytes) and neutrophil elastase (neutrophils) as detailed in **table 2(b)**.

#### 2:3.1. Dewaxing and antigen retrieval

Paraffin sections were heated to 60°C for 35 minutes, deparaffinized in xylene, and rehydrated in a 100% ethanol 2 x 5mins, 95% ethanol 2 x 5mins, 90% ethanol 1 x 5mins and 70% ethanol 1 x 5mins. Endogenous peroxidase activity was quenched using 0.5%

hydrogen peroxide in methanol. Sections were washed in Phosphate Buffered Saline (PBS) and, when required, the antigen was retrieved by microwaving in a pressure cooker (Lakeland Plastics Ltd, Cumbria, UK) at full power for 5 min in citrate buffer (10mM, pH 6.0) or by enzymatic digestion with a 0.1% (w/v) trypsin (Sigma, Poole, Dorset, UK) solution in 50mM Tris buffer (pH 7.6) containing 0.1% (w/v) calcium chloride, for 10 minutes at 37°C (refer to **Table 2b**).

### **2:3.2. ABC method**

All sections to be incubated with antibodies against interleukins were washed in PBS with 0.1% saponin (Sigma) added (to permeabilise the cell membrane) then blocked with 20% rabbit/ 20% human serum; sections to be incubated with CD45, CD68, CD20 and neutrophil elastase were washed in PBS then blocked in 20% horse/ 20% human serum for 30 minutes at room temperature and incubated for 16 hr at 4°C with the 1°antibody (**Table 2b**) diluted either in 2% rabbit serum with 0.1% saponin added (cytokines) or 2% horse serum (leukocyte markers). Sections were washed in either PBS/0.1% saponin or PBS then incubated for 30 min with biotinylated rabbit anti-goat (Vector Laboratories) (cytokines) or biotinylated horse anti-mouse (Vector Laboratories) (leukocyte markers) diluted 1:200 in 2% rabbit serum in PBS/0.1% saponin or 2% horse serum in PBS both with 5% human serum added. Next, sections were washed in PBS/0.1% saponin or PBS then incubated with avidin DH/ biotinylated horseradish peroxidase H reagent (Vector Laboratories) in PBS for 30 min before final washing. The antigen was localized using 1mg/ml diaminobenzidine tetrahydrochloride (DAB/Sigma UK), 0.2% H<sub>2</sub>O<sub>2</sub> in 50mM Tris. HCl, pH 7.6 and appeared as a brown end-product. Sections were counter stained with Harris haematoxylin (Sigma). Negative controls included slides incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG<sub>1</sub> *Aspergillus niger* glucose oxidase (Dako), an enzyme that is neither present nor inducible

in mammalian tissues. Tonsillar tissue was used as positive controls for all primary antibodies used. The specificity of each of the anti-cytokine antibodies had previously been verified by the manufacturer using enzyme linked immunosorbent assay and western blotting. The manufacturer stated there was no cross-reactivity with over one hundred other cytokines tested.

**Table 2b. Primary antibodies used for immunocytochemistry.**

Antibody	Clone	Pretreatment	Dilution	Source
<b>IL-1<math>\beta</math></b>	Polyclonal	Microwave	1:50	R + D (AF-201-NA)
<b>IL-6</b>	Polyclonal	Microwave	1:150	R + D (AF-206-NA)
<b>IL-8</b>	Polyclonal	Microwave	1:25	R + D (AF-208-NA)
<b>TNF<math>\alpha</math></b>	Polyclonal	Microwave	1:150	R + D (AF-210-NA)
<b>CD45</b>	2B11 + PD7/26	Microwave	1:100	DAKO (M 0701)
<b>CD68</b>	PG-M1	Trypsin	1:300	DAKO (M 0876)
<b>CD3</b>	Polyclonal	Microwave	1:500	DAKO (A 0452)
<b>CD20</b>	L26	Microwave	1:500	DAKO (M 0755)
<b>Neutrophil elastase</b>	NP57	Nil	1:150	DAKO (M 0752)

#### **2:4. Quantification of inflammatory cell density.**

Following immunocytochemistry with the appropriate white cell markers leucocyte density was quantified in cervix, fetal membranes and myometrium before and during

labour at term. The leucocytes were identified by histological analysis. For each sample the number of leucocytes, macrophages, neutrophils, T-lymphocytes and B-lymphocytes in ten randomly (six in cervix) selected high power fields (x 400 objective magnification) were counted by two observers who were blinded to the specimen details. The area for each highpowered field was  $0.23\text{mm}^2$ . White cells within the blood vessels were not included in the counts. Within the samples of fetal membranes the leucocyte subpopulations were quantified in amnion, chorion and decidua. The means for each observer were calculated and compared, there was less than 5% difference in counts between observers. The median density of positive cells for each specimen was calculated and statistical analysis of the medians were performed using Kruskal-Wallis, a  $p$  value of less than 0.05 was considered significant.

#### **2:5. Northern analysis.**

Northern blotting followed by hybridisation analysis is the basic technique employed to study steady-state RNA levels. It can be employed for quantitative analysis for example to examine tissue distribution and abundance. It involves a radioactive probe and RNA that has been immobilised on a filter membrane. The hybridisation is between complementary bases in the RNA and the probe. The RNA is isolated from tissue samples and after electrophoresis the RNA is transferred to the membrane and probed. After probing has been completed autoradiography is carried out with X-ray film and the intensity of the bands compared with a "housekeeping gene" i.e. human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the ratio determined. It is important to set aside all reagents and equipment solely for RNA extraction and analysis. The most common source of exogenous RNase contamination are the hands, therefore all handling of reagents and equipment should be carried out while wearing gloves. To reduce the activity of

endogenous RNases that are released during cell lysis, all manipulations should be completed quickly, on ice where possible. RNA should be stored in small aliquots at  $-70^{\circ}\text{C}$ , repeat freeze-thawing of samples should be avoided.

### **2:5.1. RNA isolation using Trizol<sup>®</sup>.**

The frozen tissue samples were ground using a mortar and pestle and the tissue transferred to a universal containing the appropriate amount of Trizol<sup>®</sup> (Life Technologies 15596-026) [1ml per 50-100mg of tissue]. Trizol<sup>®</sup> disrupts cells and dissolves the cell components freeing the RNA. The ground tissue was homogenised in Trizol<sup>®</sup> using a homogeniser (Polytron<sup>®</sup>, Novara) for 2 bursts of 30secs at speed 5. The homogenate was aliquoted in 1ml amounts into microcentrifuge tubes and incubated at RT for 5 minutes to permit complete dissociation of nucleoprotein complexes. To separate the solution into an aqueous phase and an organic phase 0.2ml of chloroform was added per 1ml of Trizol<sup>®</sup> homogenate. The tubes were shaken vigorously for 15 seconds until the solution turned a cloudy red colour. The samples were then incubated at room temperature (RT) for 3 minutes, followed by centrifugation at 13,000rpm (10,000g) for 15 minutes at  $4^{\circ}\text{C}$ . The upper aqueous phase of the sample was transferred to a fresh tube. The RNA was then precipitated from the aqueous phase by mixing with isopropyl alcohol (0.5ml of isopropyl alcohol per 1ml of Trizol<sup>®</sup> used in the initial homogenisation). The samples were then incubated at RT for 10 minutes then centrifuged at 13,000rpm for 15 minutes at  $4^{\circ}\text{C}$ . After this centrifugation step, 1ml of 75% ethanol was added to each RNA pellet sample, each sample was then vortexed. At this stage the samples were stored at  $-70^{\circ}\text{C}$  until quantification

### **2:5.2. Quantification of RNA.**

RNA samples were centrifuged at 10,000rpm for 5 minutes at 4°C, the ethanol was poured off and the tubes gently tapped constantly observing the pellet at the bottom of the tube. The tubes were inverted and placed on filter paper and the pellets dried at RT for 20-30 minutes. An appropriate amount of diethylpyrocarbonate-treated deionised distilled water (DEPC DDW) was added to each pellet (to a large pellet add 40ul DEPC DDW and a small pellet around 15ul DEPC DDW). The samples were vortexed, spun down quickly and incubated at 65°C for 5 minutes in a dri-block (Techne). Samples were once more vortexed, spun down and incubated for a further 5 minutes at 65°C. All dissolved pellets from the same sample were combined in one tube and samples kept on ice at all times. The quantity of RNA present in the samples was calculated using a spectrophotometer (UltrospecIII, Amersham Pharmacia Biotech). Absorbance readings were taken at 260nm and 280nm. An aliquot of each sample was diluted in distilled water and placed in a quartz cuvette. The spectrophotometer was first blanked with distilled water alone. The reading at 260nm gives an indication of the amount of nucleic acid, in this case RNA, is present in each sample, the reading at 280nm gives an estimation of the amount of protein present. The ratio of the 260/280 readings indicates the purity of the sample, for RNA a ratio of >2.0 being optimum. The 260nm reading was used to calculate the amount of RNA present in each sample, 1 OD unit at 260nm is equivalent to 40mg RNA.

### **2:5.3. RNA gel.**

To determine the integrity of the RNA extracted from the tissue samples 10µg amounts of each sample were run out in a horizontal gel tank (Amersham Pharmacia Biotech) in a gel containing 1.2g agarose (Sigma) with 73ml DDW and 10ml 10 x MOPS (0.2 mol/L 3-[N-morpholino] propane-sulphonic acid; 0.05 mol/L Na acetate, pH 7.0; 0.01 mol/L Na<sub>2</sub>

ethylenediaminetetra-acetic acid) added. After heating the agarose solution in a microwave for 2mins 30secs at power level 7, 17ml formaldehyde was added, and allowed to cool. Then 60ml of gel solution was poured onto a sealed gel plate with comb in place and allowed to set. The comb was removed and the gel placed in a G-100 gel tank (Amersham Pharmacia Biotech) and filled with 1 x MOPS. RNA samples were removed from the -70°C freezer and thawed on ice. The samples were then incubated at 65°C for 5 minutes in a dry block and 10µl of RNA loading buffer (Sigma R4268) added to each sample. Samples were further heated at 65°C for 10 minutes, loaded onto the gel with care and run at 60V using a Pharmacia LKB – GPS 200/400 power supply for 2-2.5 hours.

#### **2:5.4. Blotting gel (RNA transfer).**

RNA was transferred onto Hybond-N (Amersham Pharmacia Biotech) nylon membranes by blotting in approximately 300ml of 20 x SSC (3mol/L NaCl, 0.3mol/L NaCit). Briefly the gel was placed on 20 x SSC soaked filter paper sheets, all the air bubbles underneath the gel were removed using a 10ml pipette. The nylon membrane was cut to the same size as the gel and placed on top of the gel. The membrane was protected by a sheet of filter paper. All around the gel was covered with clingfilm to prevent the 20 x SSC from evaporating, 25 blotting sheets and a weighted bottle were placed on top of the gel. The RNA was allowed to transfer overnight. After removing the clingfilm, blotting sheets and filter paper the membrane was handled carefully, with only the edges of membrane being touched and was rinsed in 5 x SSC. The RNA was then crosslinked onto the nylon membrane (RNA side up) using ultra violet radiation in the Stratalinker (40s at 1.2x10<sup>5</sup> mjoules). Small cuts were made at the 18S and 28S marker area on the nylon membrane while being viewed on a transilluminator. The nylon membrane was stored at -20°C in vacuum - sealed plastic sheeting.

**2:5.5. Random priming of cDNA probes.**

cDNA (50ng) was required for each reaction (50ul total reaction volume). The 50ng aliquot of DNA was made up to 47ul volume with TE (Tris EDTA) buffer, the lid of the microcentrifuge tube was pierced and the tube boiled for 5 minutes, placed on ice for 5 minutes and briefly centrifuged. The denatured DNA was added to a tube containing beads [Ready -To- Go] DNA labelling beads (Amersham Pharmacia Biotech cat no.27-9240-01). The tube was taken behind a protective screen and 3ul  $\alpha^{32}P$  dCTP (ICN Flow) was added. The tube contents were mixed by gentle pipetting, transferred to a perspex box and then incubated for 30 minutes at 37°C in a waterbath. G50 sephadex (3-4ml) was added to 10ml pipette (broken at 6ml stage), stoppered with glass wool and TE buffer carefully layered on the top of the sephadex. A 50ul pipette was used to add the probe to the sephadex column. Approximately 1.2ml of buffer was collected before the labelled probe came through the sephadex column. The labelled probe was collected when the reading on the Geiger counter entered the green area on the scale, as the collection progressed the reading on the monitor left the scale then returned to below the green area. The collected probe was placed on ice, boiled for 5 minutes and put on ice for a further 5 minutes. The probe was then added to the prehybridising filters using a syringe and needle.

**2:5.6. Prehybridisation and hybridisation of nylon membranes.**

Hybridisations were performed in a Techne Hybridiser HB-ID. Ultrahyb (AMS Biotechnology, Oxon, UK) was heated at 68°C for at least 1hour before being added to the appropriate nylon membrane. Approximately 12-14ml of Ultrahyb was added to the hybridisation tube. The nylon membrane was placed in the hybridisation tube with the RNA side facing the centre of the tube. Any air bubbles were removed very gently using a

10ml pipette. The hybridisation tube was then placed in the hybridiser at 42°C for at least 30 minutes prior to the addition of the radiolabelled probe. After adding the radiolabelled probe the nylon membranes were hybridised at 42°C overnight for approximately 18hours.

#### **2:5.7. Washing nylon membranes.**

The nylon membranes were washed in approximately 15ml of 1 x SSC + 0.1% SDS (sodium dodecyl sulphate) for 20 min at 65°C then washed further in 0.5 x SSC + 0.1% SDS for 20 min at 65°C. If necessary, an additional wash was carried out in 0.1 x SSC + 0.1% SDS for 20 mins at 65°C. Membranes were vacuum - sealed in plastic sheeting.

#### **2:5.8. Autoradiography and statistical analysis.**

Autoradiography was carried out with Fuji X-ray film. The probed membranes were placed in cassettes, X-ray film was positioned over the membranes and placed in a -70°C freezer from between 4hrs-10 days. After digital scanning the intensity of the bands on the autoradiographs for each of the cytokines undergoing northern analysis was compared with GAPDH and the ratio was determined using Bio-Rad Multi-Analyst/PC 1.1. Statistical analysis of band intensity for northern analysis was carried out using the Kruskal-Wallis test with the Mann-Whitney U test as a post hoc test. A *P* value less than 0.05 was considered significant. These ratios are not absolute values for molecular expression but are ratios of assay parameters and are therefore treated non-parametrically.

### **2:5.9. Stripping nylon membranes.**

All probed membranes were stripped in Tris/EDTA/Denhardt's (2ml 50mM Tris; 200 $\mu$ l 0.5M EDTA; 200 $\mu$ l 50 x Denhardt's made up to 100ml with distilled water) solution for 2 hrs at 75°C in a hybridisation oven. Stripped membranes were autoradiographed to check if they had stripped well.

The stripped membranes were then probed again with the housekeeping gene GAPDH and autoradiography was repeated.

### **2:6. Amplification by PCR of cDNA.**

The polymerase chain reaction (PCR) was first described in 1985 by Saiki R.K., and is a method of amplifying a segment of DNA that lies between two regions of a known sequence. In the PCR, two primers (short single stranded DNAs) are used that are complementary to opposite strands of the DNA sequence to be amplified. After heat mediated denaturation of the template DNA, the primers attach to their respective sequences (annealing) on the template DNA and a DNA polymerase synthesises a complementary strand in the 5 (prime) to 3 (prime) direction (extension). Each run of denaturation, annealing and extension is known as a cycle. With each cycle the quantity of the template DNA sequence amplified doubles. The optimum temperature at which each of these steps (denaturation, annealing and extension) progresses varies, so the reaction is best carried out in a thermal cycler, which automatically makes the temperature changes necessary. Template DNA (stimulated THP1 cells ECALL no. 88081201) was amplified for the interleukins IL-1 $\beta$ , IL-6, IL-8 and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ). The cytokine oligonucleotide PCR primers were designed and product size and sequence validated using

the GENBANK database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The primer sequences used are shown in **Table 2(c)**

**Table 2(c). Cytokine primer sequences**

Cytokine primer	Sequence (All 5' - 3')	Product
<b>IL-1<math>\beta</math> primer F</b>	ATG GCA GAA GTA CCT AAG CTC	810 base pairs
<b>IL-1<math>\beta</math> primer R</b>	TTA GGA AGA CAC AAA TTG CAT GGT GAA CTC AGT	
<b>IL-6 primer F</b>	ATG AAC TCC TTC TCC ACA AGC	639 base pairs
<b>IL-6 primer R</b>	CTA CAT TTG CCG AAG AGC CCT CAG GCT GGA CTG	
<b>IL-8 primer F</b>	ATG ACT TCC AAG CTG GCC GTG	302 base pairs
<b>IL-8 primer R</b>	TTA TGA ATT CTC AGC CCT CTT CAA AAA CTT CTC	
<b>TNF <math>\alpha</math> primer F</b>	ATG AGC ACT GAA AGC ATG ATC	702 base pairs
<b>TNF <math>\alpha</math> primer R</b>	TCA CAG GGC AAT GAT CCC AAA GTA GAC CTG CCC	

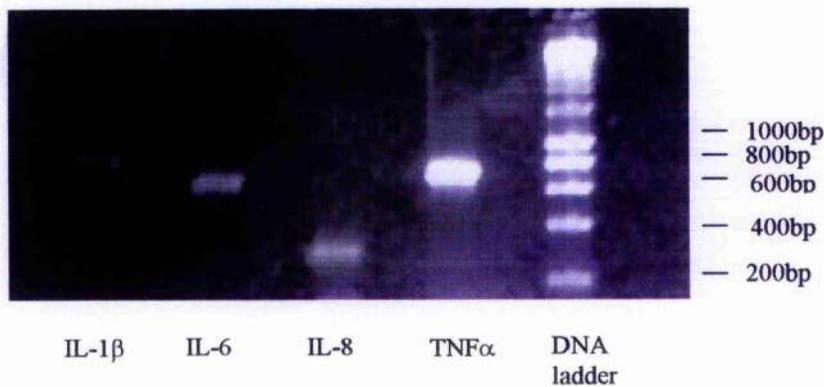
(where F = forward, R = reverse)

All PCRs were carried out on a Techne Genius PCR machine (Scotlab, Lanarkshire). The Taq polymerase used in all PCRs was supplied from Advanced Biotechnologies (Surrey, UK). Briefly 100ng of template DNA was amplified using 0.5 units of Taq polymerase in 1 x Buffer IV (200mM  $[\text{NH}_4]_2\text{SO}_4$ , 750mM Tris-HCL pH 8.8, 0.1% (v/v) Tween 20 (Bioline), 1.5mM  $\text{MgCl}_2$  (Bioline), 200mM dNTPs (dATP, dGTP, dTTP, dCTP [Life Technologies]), 0.12 $\mu\text{M}$  of each primer made up to a final volume of 25 $\mu\text{l}$  with sterile distilled water. This was subjected to 5 minutes at 95°C, followed by 30 cycles of 95° for 1 minute, 60°C for 1 minute (56°C for IL-8 and TNF $\alpha$ ) and 72°C for 2 minutes with a final extension of 72°C for 10minutes. A 10 $\mu\text{l}$  aliquot of the PCR product was mixed with

5 $\mu$ l of loading buffer (50% glycerol, 50% 10 X TBE with orange G) and run out on a 2% agarose (Invitrogen Life Technologies, Paisley, Scotland) gel containing 5 $\mu$ l of ethidium bromide 10mg/ml (Sigma, UK) in a horizontal gel tank (Anachem, Bedfordshire, UK) in 1 x TBE (Tris base, boric acid, EDTA) with a constant current of approximately 50mAmp for 1hr. The gel was photographed on a UV transilluminator (Spectroline®, model TC-312A, Genetic Research Instrumentation, Essex, England).

### Figure 2b. Gel electrophoresis of PCR products

Product sizes were IL-1 $\beta$  810 base pairs (bp), IL-6 639bp, IL-8 302bp and TNF $\alpha$  702bp.



#### 2:6.1. Purification of PCR products.

The cytokine (IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ ) PCR products were run out on a 2% low melting temperature agarose (Nu Sieve) gel in 1x TAE (TRIS, EDTA + acetic acid) buffer with 5 $\mu$ l of ethidium bromide 10mg/ml added. To the PCR product 5 $\mu$ l of orange G (Sigma) 200 $\mu$ g/ml in 50% glycerol/TAE was added then loaded onto the gel and run for two hours at approximately 50mAmps, DNA ladder 2 $\mu$ l (Bioline, UK.) was also loaded onto the gel. The DNA bands in the gel were visualized on a UV transilluminator and excised at the appropriate size using a scalpel. Purification of the DNA from the agarose was achieved

using Wizard PCR Preparations DNA purification system (Promega, UK.) The agarose slices were transferred to a 1.5ml tube and incubated at 70°C until the agarose was completely melted, 1 ml of resin from the purification kit was added to the melted agarose and mixed thoroughly for 20 seconds but not vortexed. For each PCR product one Wizard™ Minicolumn was used. The plunger from a 3 ml disposable syringe was removed and set aside, the syringe barrel was attached to the Luer-Lok® extension of each minicolumn and the resin/DNA mix pipetted into the syringe barrel. The syringe plunger was gently inserted into the syringe barrel and the slurry carefully pushed into the minicolumn. The syringe was detached from the column and the plunger then removed, the syringe barrel was reattached to the minicolumn and 2ml of 80% isopropanol (BDH, Poole, UK.) pipetted into the syringe and gently pushed through to wash the column. After removing the syringe the minicolumn was transferred to a 1.5ml microcentrifuge tube and centrifuged at 10,000g to dry the resin. The minicolumn was then removed to a new microcentrifuge tube and 50µl of TE buffer added, after 1 minute the minicolumn was centrifuged for 20 seconds at 10,000g to elute the bound DNA fragment.

#### **2:6.2. DNA quantitation.**

To calculate the amount of DNA yielded from the Wizard prep an absorbance reading was taken at both 260nm and 280nm on a Cecil Bioquest CE2501 spectrophotometer. An aliquot of each sample was diluted in sterile distilled water (1:500) and placed in a quartz cuvette. The spectrophotometer was first blanked with distilled water alone before the samples were analysed. The reading at 260nm gives an indication of the amount of nucleic acid, in this case DNA, that is present in each sample, the reading at 280nm an estimation of the amount of protein present. The ratio of the 260/280 readings indicates the purity of the sample, for DNA a ratio of approximately 1.8 is optimum. The 260nm reading was

used to calculate the yield, 1 OD unit at 260nm is equivalent to 50 $\mu$ g/ml of double stranded DNA.

### **2:7. Image analysis.**

An Olympus BX50 microscope equipped with x4, x10, x20, x40 lenses, connected to a 3-CCD colour camera (JVC) was used for digital image capture. Computer visualisation of the images was achieved with the image analysis program Image-Pro Plus 4.0. The autoradiograph bands were scanned using an Epson GT 9500 scanner and the images imported to Adobe Photoshop 4.0 or 5.5 using the TWAIN 32 interface program. The final autoradiograph images are composite images which have been manually aligned using Adobe photoshop software.

### **2:8. Buffers and solutions.**

#### **Buffers for immunocytochemistry**

##### **Phosphate Buffered Saline**

10mM sodium phosphate (Sigma), 120mm sodium chloride (BDH) in distilled water, pH 7.5.

##### **Citrate Buffer**

10mM anhydrous citric acid (BDH) in distilled water, pH 6.0.

##### **Tris ([hydroxymethyl]aminomethane) HCl Buffer**

50mM Tris 7-13 (Sigma) in distilled water, pH 7.2 adjusted with concentrated HCl.

### **Solutions and Buffers for Northern Analysis**

#### **Distilled Deionised Water (DDW)**

All solutions and buffers were prepared using DDW obtained from a Milli Q Plus water purification system purchased from Millipore S.A. (Molsheim, France). DEPC (diethylpyrocarbonate) 500 $\mu$ l was added to 500ml of DDW which was then autoclaved and stored at 4°C.

#### **Concentrated (20x) sodium saline citrate (SSC)**

3M sodium chloride (BDH), 0.3M sodium citrate (BDH) in DDW, pH 7.0 adjusted with concentrated hydrochloric acid (Sigma). The solution was autoclaved and stored at 4°C.

#### **EDTA**

0.5M EDTA in DDW, pH 8.0 adjusted with 10N sodium hydroxide (BDH). The solution was autoclaved and stored at 4°C.

#### **TrisEDTA (ethylenediaminetetra-acetic acid [TE]) electrophoresis buffer**

2.42g Tris HCl (Sigma) was dissolved in 4ml 0.5M EDTA (pH 8.0) and made up to 2L with DDW, the pH was adjusted to pH 8.0 autoclaved and stored at 4°C.

#### **Concentrated (10x) MOPS (3-[N-morpholino] propanesulphonic acid)**

41.2g MOPS (Sigma) was dissolved in 800ml of 0.1M sodium acetate (BDH), the pH was adjusted to pH 7.0 with 10N sodium hydroxide. 10ml of 0.5M EDTA was added and the

final volume adjusted to 1L with DDW. The MOPS buffer solution was autoclaved and stored at 4°C.

#### 50x Denhardt's

1g of polyvinylpyrrolidone (PVP, Sigma), 1g Ficoll (Amersham Pharmacia Biotech) and 1g BSA (fraction V, Sigma) was added to 80ml DEPC treated DDW. After stirring this solution was made up to 100ml and aliquoted in 5ml batches and stored at -20°C.

#### **Solutions and buffers for cDNA preparation**

##### TAE (TrisAcetateElectrophoresis) Buffer

50 x TAE, 242g Tris base (Sigma) in 500ml H<sub>2</sub>O, 100ml of 0.5M EDTA (pH 8.0), 57.1ml of glacial acetic acid (BDH), was adjusted to 1 Litre with H<sub>2</sub>O. The buffer was stored at room temperature and used at 1 x.

##### TBE (TrisBorateElectrophoresis) Buffer

5 x TBE, 136.25g Tris base (Sigma), 69.7g boric acid (BDH), 18.75g EDTA (BDH) was made up to a final volume of 5 Litres with H<sub>2</sub>O and the pH adjusted to 8.3 with 5M NaOH (BDII)

## **Chapter 3.**

### **Results.**

### 3:1. Immunocytochemistry.

#### IL-1 $\beta$

In the myometrium (n=10 IL + NIL) and cervix (n=8 IL + NIL), IL-1 $\beta$  was localized mainly in leukocytes in samples taken in labour (**Plate 3a and b**). Faint staining for IL-1 $\beta$  was demonstrated within myocytes in the myometrium. There was a significantly greater ( $p < 0.001$  [median 30.05 (IQ range 8.1-55.6)]) density of positively staining leukocytes in myometrial biopsies sampled after the onset of labour compared with those sampled before labour (median 3.38 [IQ range 1.7-4.1]). In cervical biopsies taken prior to the onset of labour there was little IL-1 $\beta$  staining (**Plate 3a**) whereas in the labouring cervical biopsies the plentiful leukocytes stained positively for IL-1 $\beta$  (**Plate 3b**). In fetal membranes (n=10 IL + NIL), IL-1 $\beta$  was located in leukocytes and the stromal cells of the decidua (**Plate 3c**).

#### IL-6

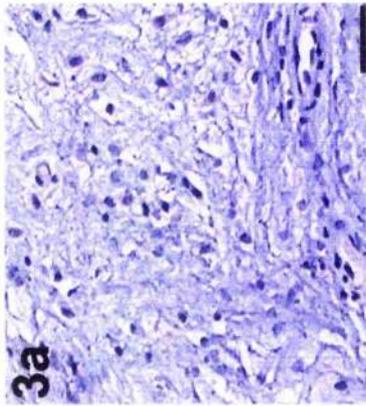
IL-6 localization was limited to a subpopulation of leukocytes within the myometrium (n=10 IL + NIL). In the cervix (n=8 IL + NIL), IL-6 was restricted to a subpopulation of leukocytes and glandular epithelium and surface epithelium (**Plate 3d and e**). These leukocytes were multilobed in appearance indicative of being neutrophils. In fetal membranes (n=10 IL + NIL), IL-6 was located in decidual stromal cells, mesenchymal cells, infiltrating leukocytes and extravillous trophoblast (**Plate 3f**). The density of leukocytes staining positively for IL-6 appeared greater in labouring compared with non-labouring myometrium and cervix (**Plate 3d and e**) consistent with the IL-1 $\beta$  pattern.

**IL-8**

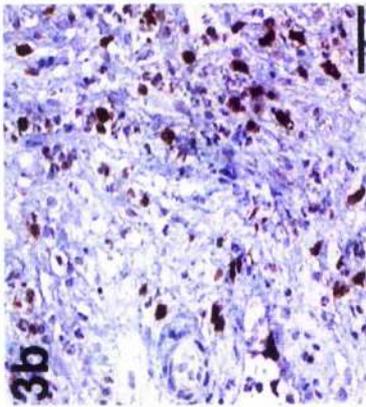
In myometrium (n=10 IL + NIL), immunostaining for IL-8 was limited to infiltrating leukocytes in labouring tissues. These cells were not observed in samples taken from non-labouring tissues (**Plate 3g and h**). In cervix (n=8 IL + NIL), IL-8 was identified in surface epithelium, glandular epithelial cells, stromal cells and leukocytes. Comparing labouring with non-labouring tissues again there was a significant difference ( $p < 0.04$  [median 22.3 (IQ range 8.6-43.8)]) in the density of leukocytes in cervix biopsies taken after the onset of labour (median 10.15 [IQ range 4.7-13.1]). In fetal membranes (n=10 IL + NIL), IL-8 staining was restricted to leukocytes in the chorion. The amnion showed no staining (**Plate 3i**). In the labouring biopsies, IL-8 was localised in decidual stromal cells. IL-8 was also seen in the mesenchymal cells between amnion and chorion, where present.

**TNF- $\alpha$** 

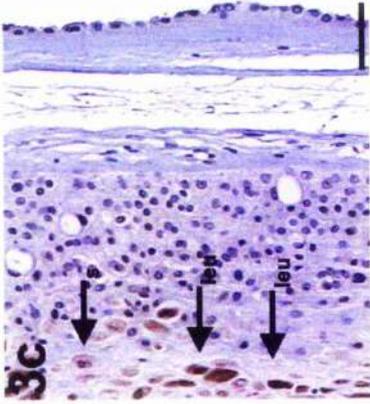
Immunostaining for TNF $\alpha$  was confined to a small subpopulation of leukocytes in labouring myometrium (n=10 IL + NIL) (**Plate 3k**). There was little TNF- $\alpha$  staining in non-labouring samples (**Plate 3j**). The morphology of the leukocytes shown to stain for TNF- $\alpha$  indicated that these cells are possibly macrophages. In cervix (n=8 IL + NIL) TNF- $\alpha$  was located to glandular epithelium, surface epithelial cells and leukocytes. In fetal membranes (n=10 IL + NIL), staining for TNF- $\alpha$  was detected in decidual stromal cells, mesenchymal cells, and extravillous trophoblast (**Plate 3l**). As found with IL-1 $\beta$ , IL-6 and IL-8 the density of positive staining leukocytes for TNF $\alpha$  was greater in labouring versus non-labouring tissues.



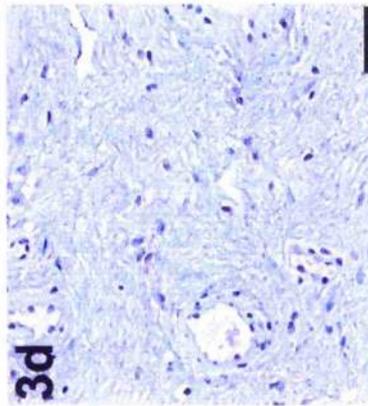
**Plate 3a.** Cervical biopsy not in labour shows little staining for IL-1 $\beta$ .



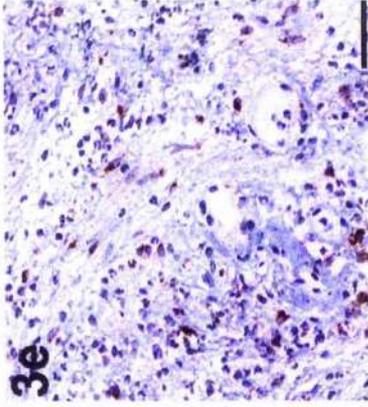
**Plate 3b.** Cervical biopsy in labour shows IL-1 $\beta$  within abundant leukocytes.



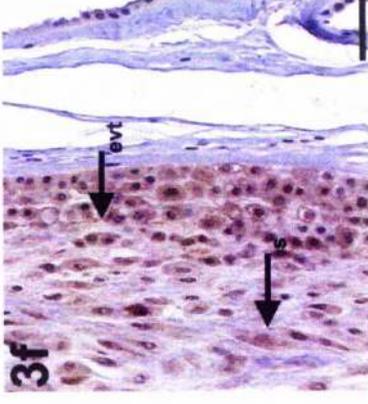
**Plate 3c.** IL-1 $\beta$  is localised to leukocytes (leu) decidual stromal cells (s).



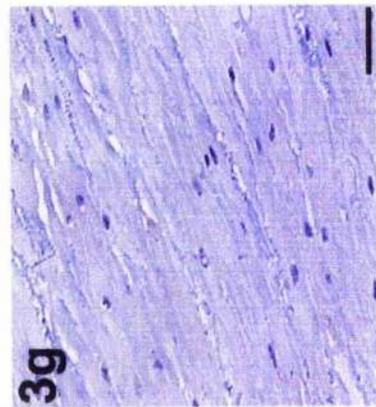
**Plate 3d.** In cervical biopsy not in labour IL-6 was restricted to a sub-population of leukocytes



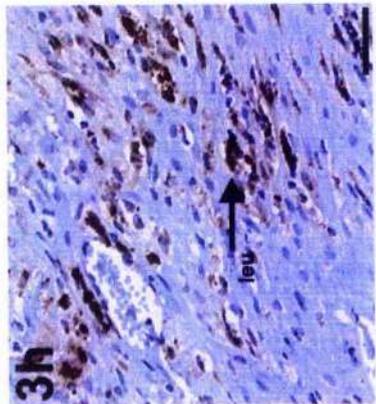
**Plate 3d.** In cervical biopsy not in labour IL-6 was restricted to a sub-population of leukocytes which were greater in number compared with cervix not in labour.



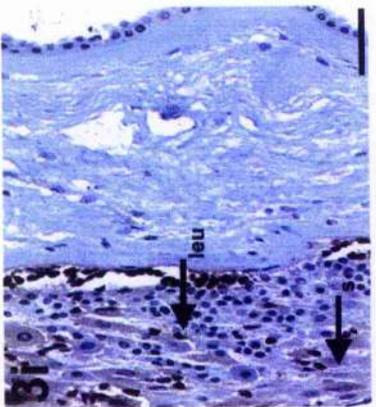
**Plate 3f.** Fetal membranes show IL-6 in decidual stromal cells (s) and extravillous trophoblast (evt).



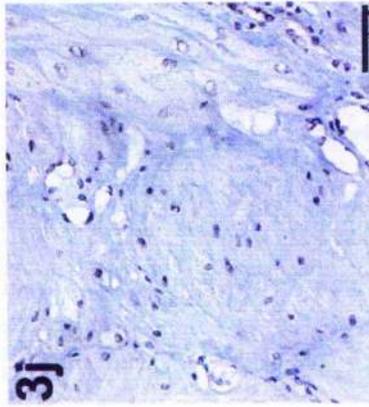
**Plate 3g.** In myometrium not in labour IL-8 was not identified.



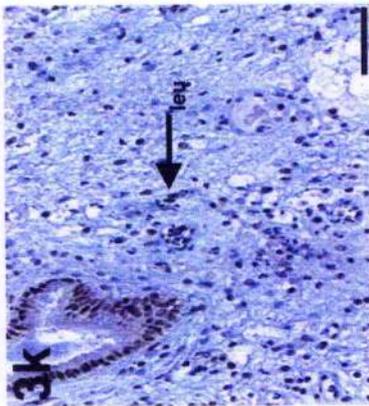
**Plate 3h.** In myometrium in labour IL-8 was present within leukocytes (leu).



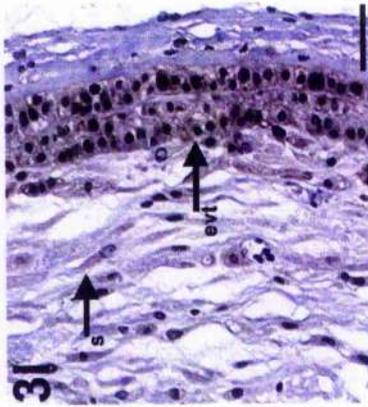
**Plate 3i.** In fetal membranes IL-8 was present in decidua stroma cells (s) and invading leukocytes (leu).



**Plate 3j.** TNFα staining was absent in non-labouring myometrium.



**Plate 3k.** Labouring cervix showing TNFα in leukocytes (leu).



**Plate 3l.** In fetal membranes TNFα is present in decidua stroma cells (s) and extravillous trophoblast (cvt).

Scale bars = 50µm.

### 3:2. Northern blotting analysis.

Northern blotting confirmed the presence or absence of mRNA for IL-1 $\beta$ , IL-6 and IL-8 in amnion (n=6)(Fig 3a), choriodecidua (n=6)(Fig 3b), cervix (n=6) (Fig 3c) and myometrium(n=6) (Fig 3d). TNF $\alpha$  mRNA expression was below the limits of detection of our assay. Comparison of the ratios of mRNA between labouring and non-labouring uterine tissues for IL-1 $\beta$ , IL-6 and IL-8 are shown in Figures 3e, 3f and 3g. Where cytokine mRNA was absent it was considered to be at the lower limit of detection of the assay for the purpose of calculating the ratio of expression in labouring versus non-labouring uterine tissues. All control genes are difficult to select, in the literature most studies use GAPDH but subsequent studies in our laboratory have shifted to using 18s as GAPDH may be differentially expressed in different tissues. Levels of RNA varied mostly in amnion and cervix as these tissues were technically difficult to process for RNA extraction.

#### Fetal membranes.

The expression of IL-1 $\beta$  (p<0.02 [median 0.21 (IQ range 0.12-0.35)]) and IL-8 (p<0.01 [median 0.24 (IQ range 0.10-0.40)]) was significantly greater in amnion following spontaneous labour (Figures 3a, 3e and 3g). The expression of IL-6 (p<0.05 [median 1.42 (IQ range 1.11-2.06)]) and IL-8 (p<0.02 [median 0.89 (IQ range 0.38-1.62)]) was significantly greater in choriodecidua biopsies sampled after the onset of labour (Figures 3b, 3f and 3g). In the amnion and choriodecidua the expression of TNF $\alpha$  mRNA was beneath the limits of detection of the assay used.

**Cervix.**

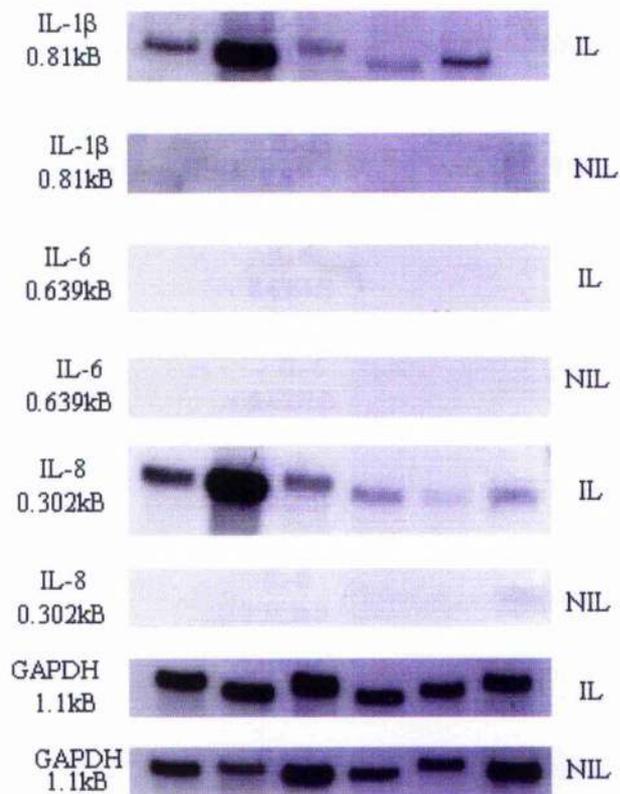
In cervix the expression of IL-1 $\beta$  ( $p < 0.02$  [median 1.45 (IQ range 0.69-2.43)]), IL-6 ( $p < 0.05$  [median 1.35 (IQ range 0.65-2.5)]) and IL-8 ( $p < 0.02$  [median 1.30 (IQ range 0.32-2.40)]) was significantly greater in labouring tissues compared with non-labouring tissues **Figures 3c, 3e, 3f and 3g**. TNF $\alpha$  mRNA expression was below the limits of detection of the assay used.

**Myometrium.**

In myometrium the expression of IL-1 $\beta$  ( $p < 0.02$  [median 0.27 (IQ range 0.12-1.42)]), IL-6 ( $p < 0.005$  [median 1.34 (IQ range 1.23-2.41)]) and IL-8 ( $p < 0.005$  [median 0.63 (IQ range 0.49-1.76)]) was significantly greater in biopsies collected following spontaneous labour compared with non-labouring biopsies **Figures 3d, 3e, 3f and 3g**. TNF $\alpha$  mRNA expression was below the limits of detection of the assay employed.

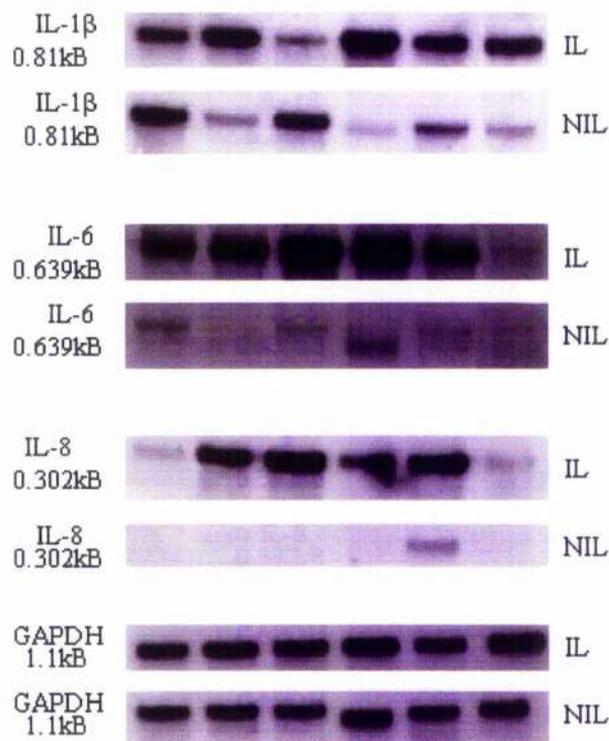
**Figure 3a. Autoradiographs of amnion samples after the onset of labour (IL) vs samples of amnion before the onset of labour (NIL).**

Northern blot hybridisation of total RNA (10 $\mu$ g per lane) measuring IL-1 $\beta$ , IL-6, IL-8 and using GAPDH as the housekeeping gene, from human amnion samples (n=6) collected before the onset of labour and after the onset of labour.



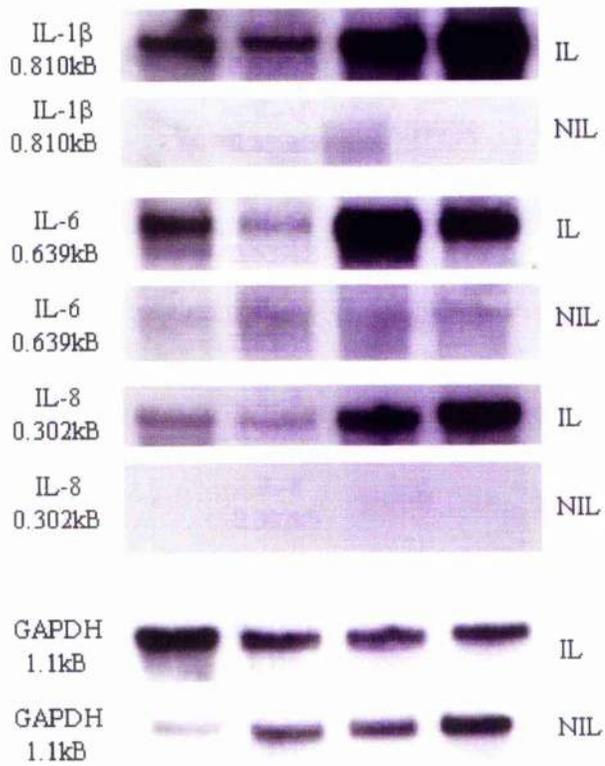
**Figure 3b. Autoradiographs of chorio/decidua samples after the onset of labour (IL) vs samples of chorio/decidua before the onset of labour (NIL).**

Northern blot hybridisation of total RNA (10 $\mu$ g per lane) measuring IL-1 $\beta$ , IL-6, IL-8 and using GAPDH as the housekeeping gene, from human chorio/decidua samples (n=6) collected before the onset of labour and after the onset of labour.



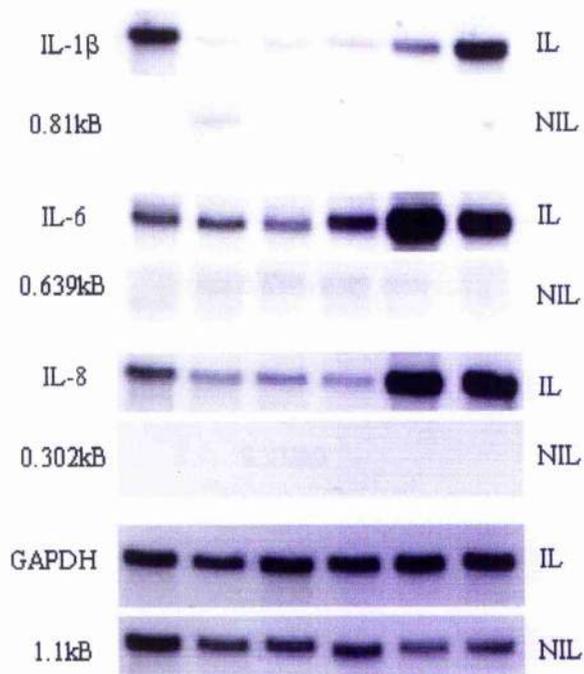
**Figure 3c. Autoradiographs of samples of cervix after the onset of labour (IL) vs samples of cervix before the onset of labour (NIL).**

Northern blot hybridisation of total RNA (10 $\mu$ g per lane) measuring IL-1 $\beta$ , IL-6, IL-8 and using GAPDH as the housekeeping gene, from human cervical samples (n=4) collected before the onset of labour and after the onset of labour.



**Figure 3d. Autoradiographs of myometrial samples after the onset of labour (IL) vs samples of myometrium before the onset of labour (NIL).**

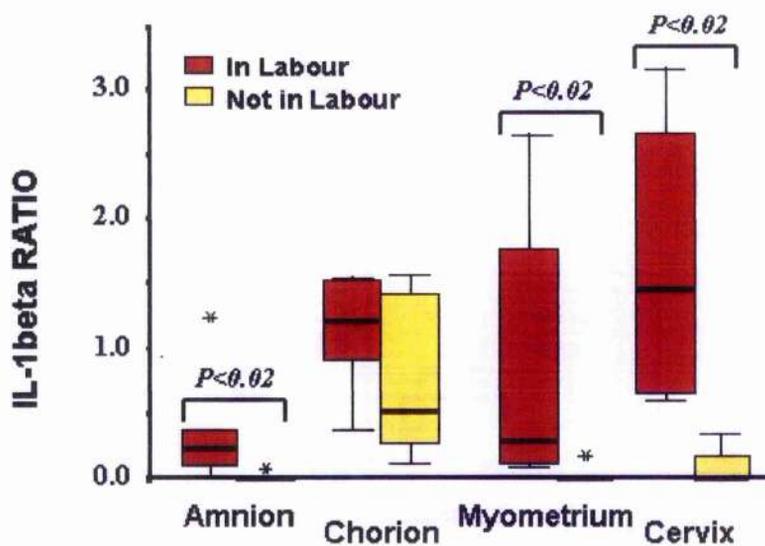
Northern blot hybridisation of total RNA (10 $\mu$ g per lane) measuring IL-1 $\beta$ , IL-6, IL-8 and using GAPDH as the housekeeping gene, from human myometrial samples (n=6) collected before the onset of labour and after the onset of labour.



**Figure 3e. Ratio of IL-1 $\beta$  mRNA/GAPDH mRNA in uterine tissues labour vs non-labour.**

*P* values show the difference between in labour vs not in labour ratios. Standard box and whisker plots showing distributions, interquartile ranges and median values.

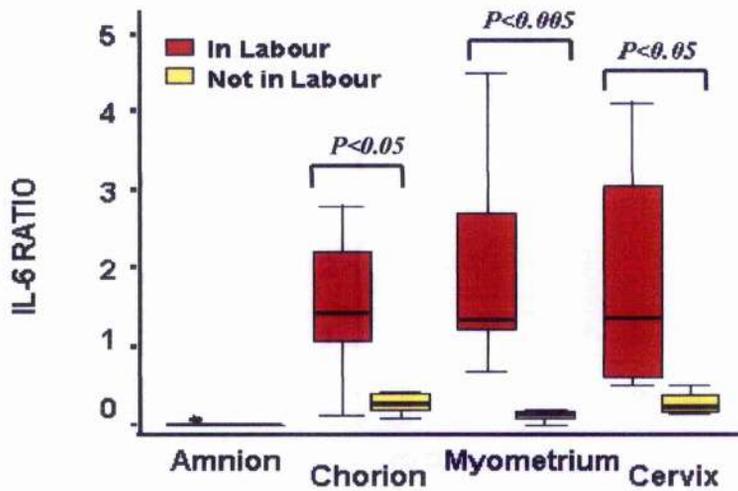
\* outliers



**Figure 3f. Ratio of IL-6 mRNA/GAPDH mRNA in uterine tissues labour vs non-labour.**

*P* values show the difference between in labour vs not in labour ratios. Standard box and whisker plots showing distributions, interquartile ranges and median values.

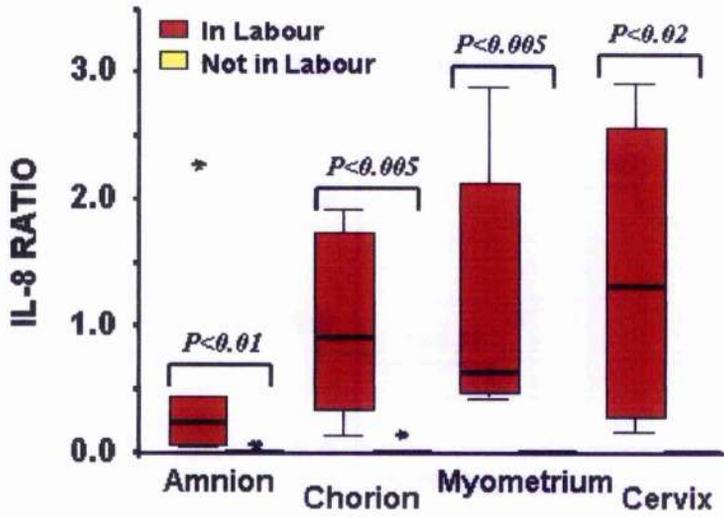
\* outlier



**Figure 3g. Ratio of IL-8 mRNA/GAPDH mRNA in uterine tissues labour vs non-labour.**

*P* values show the difference between in labour vs not in labour ratios. Standard box and whisker plots showing distributions, interquartile ranges and median values.

\* outliers



### **3:3. Inflammatory cell density.**

Inflammatory cell markers (CD45, CD3, CD20, CD68 and neutrophil elastase), for each tissue group (cervix, myometrium, amnion, chorion and decidua) comparing in labour vs not in labour per highpowered field were counted.

#### **Cervix**

The main structure of the cervical biopsies was cervical stroma. There was little epithelial tissue present mainly due to microwaving so evaluation of leukocyte numbers was carried out in the stroma. The inflammatory cell types identified by immunocytochemistry in labouring and non-labouring stroma are shown in **Table 3(a)**. Total leukocyte density and density of neutrophils (**Plates 3m and 3n**) and macrophages was greater in labouring vs non-labouring cervix. T-cell and B-cell lymphocytes were present in labouring and non-labouring cervix but there was no significant difference in density.

#### **Myometrium**

In myometrial biopsies the inflammatory cell types identified by immunocytochemistry in labouring and non-labouring myometrial tissues are shown in **Table 3(b)**. Total leukocyte density and density of T-cells, macrophages (**Plates 3o and 3p**) and neutrophils was greater in labouring vs non-labouring myometrium.

**Amnion**

Lymphocytes were few in numbers in amnion biopsies and the biopsies obtained during labour were greatly disrupted by microwaving. The inflammatory cell types identified by immunocytochemistry are shown in **Table 3(c)**. There were no significant differences in cell densities comparing labouring with non-labouring amnion.

**Chorion**

The inflammatory cell types identified by immunocytochemistry in chorion biopsies are shown in **Table 3(d)**. There were no significant differences in the densities of any of the inflammatory cell types identified.

**Decidua**

While leucocytes were present in the decidual biopsies there was no significant differences in the inflammatory cell types identified between labouring and non-labouring decidual tissues. There was a marked difference in the numbers of lymphocytes and macrophages present in decidual samples when compared with amnion and chorion samples. The inflammatory cells identified by immunocytochemistry are shown in **Table 3(e)**.

**Table 3(a).** Density of inflammatory cells in cervix biopsies before and during labour at term, median (interquartile range) per highpowered field (x 40 magnification).

Cell marker	In labour n=8	Not in labour n=8	P =
	Median (IQ range)	Median (IQ range)	
CD 45 (leukocytes)	22.3 (8.6-43.9)	10.15 (4.7-13.1)	<0.040
CD 3 (T-cells)	17.95 (8.1-29.5)	5.75 (2.3-13.2)	ns
CD 20 (B-cells)	0.2 (0-0.3)	0.0 (0-0.2)	ns
CD 68 (macrophages)	5.75 (1.5-9.7)	0.55 (0.2-0.75)	<0.001
Neutrophil elastase (neutrophils)	22.9 (0.8-54.7)	0.0(0-0)	<0.001

ns = not significant

**Table 3(b).** Density of inflammatory cells in myometrial biopsies before and during labour at term, median (interquartile range) per highpowered field (x 40 magnification).

Cell marker	In labour n=10	Not in labour n=10	P =
	Median (IQ range)	Median (IQ range)	
CD 45 (leukocytes)	30.05 (8.1-55.6)	3.38 (1.7-4.1)	<0.000
CD 3 (T-cells)	4.95 (1.7-14.3)	0.25 (0.4-0.6)	<0.000
CD 20 (B-cells)	0.16 (0-0.33)	0.0 (0-0)	ns
CD 68 (macrophages)	21.5 (14.4-34.3)	4.42 (2.2-4.8)	<0.001
Neutrophil elastase (neutrophils)	33.45 (15.7-80.9)	0.17 (0-0.2)	<0.001

ns = not significant

**Table 3(c).** Density of inflammatory cells in amnion biopsies before and during labour at term, median (interquartile range) per highpowered field (x 40 magnification).

Cell marker	In labour n=5*	Not in labour n=11	P =
	Median (IQ range)	Median (IQ range)	
CD 45 (leukocytes)	0.0 (0-0)	0.0 (0-0)	ns
CD 3 (T-cells)	0.23 (0-1.3)	0.05 (0-0.6)	ns
CD 20 (B-cells)	0.0 (0-0)	0.0 (0-0)	ns
CD 68 (macrophages)	0.0 (0-0)	0.0 (0-0)	ns
Neutrophil elastase (neutrophils)	0.0 (0-0)	0.0 (0-0)	ns

\* Amnion disrupted by microwaving

ns = not significant

**Table 3(d).** Density of inflammatory cells in chorion biopsies before and during labour at term, median (interquartile range) per highpowered field (x 40 magnification).

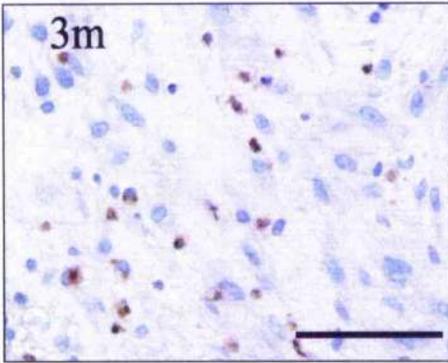
Cell marker	In labour n=9	Not in labour n=11	P =
	Median (IQ range)	Median (IQ range)	
CD 45 (leukocytes)	1.65 (0.6-14.0)	1.5 (0.4-1.7)	ns
CD 3 (T-cells)	0.4 (0-0.7)	0.8 (0.2-2.1)	ns
CD 20 (B-cells)	0.1 (0.1-0.2)	0.0 (0-0.1)	ns
CD 68 (macrophages)	0.35 (0.2-0.7)	0.2 (0.4-3.4)	ns
Neutrophil elastase (neutrophils)	2.25 (0-5.3)	0.0 (0-0.1)	ns

ns = not significant

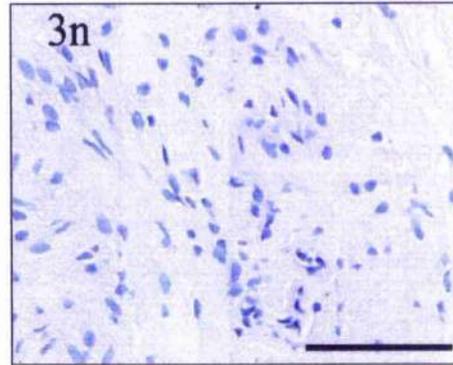
**Table 3(e).** Density of inflammatory cells in decidual biopsies before and during labour at term, median (interquartile range) per highpowered field (x 40 magnification).

<b>Cell marker</b>	<b>In labour n=9</b>	<b>Not in labour n=11</b>	<b>P =</b>
	<b>Median (IQ range)</b>	<b>Median (IQ range)</b>	
CD 45 (leukocytes)	15.4 (12.6-40.2)	18.4 (16.4-31.6)	ns
CD 3 (T-cells)	2.23 (0-9.0)	4.2 (1.8-11.4)	ns
CD 20 (B-cells)	0.1 (0.1-0.2)	0.05 (0-0.2)	ns
CD 68 (macrophages)	0.9 (0.4-1.7)	2.1 (1.5-3.6)	ns
Neutrophil elastase (neutrophils)	0.0 (0-0.1)	0.0 (0-0.7)	ns

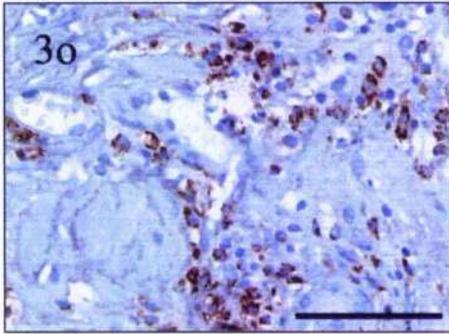
ns = not significant



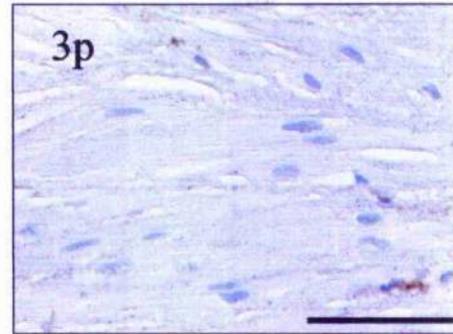
**Plate 3m.** immunolocalized neutrophils in cervix in labour.



**Plate 3n.** cervix not in labour no neutrophils immunolocalized.



**Plate 3o.** immunolocalized macrophages in myometrium in labour



**Plate 3p.** few macrophages immunolocalized in myometrium not in labour.

Scale bars = 50µm.

## **Chapter 4.**

### **Discussion.**

#### **4:1. Introduction.**

A successful conclusion to spontaneous labour and delivery of the fetus is dependent upon a number of factors: a fetus that is sufficiently mature to initiate labour and to survive outside the uterus, remodelling of extracellular matrix (ECM) in the cervix to allow cervical dilation and the progression of coordinated uterine contractions. Various pathways have been implicated in the initiation of parturition, both at term and pre-term; however all have shared concluding actions in relation to ECM remodelling and myometrial activation. One of the pathways that has been linked to the initiation and progression of labour and delivery is the inflammatory response pathway (Bowen *et al* 2002). There are several mediators involved in inflammatory responses and cytokines coordinate, to a large degree, the inflammatory response. In this study we looked at the inflammatory mediators IL-1 $\beta$ , IL-6, IL-8 and TNF $\alpha$  in cervix, myometrium and fetal membranes in-labour and before the onset of labour. We localised these inflammatory mediators in each of the tissue groups and we quantified total RNA in all tissue samples. In this study we also determined the density of leukocytes in cervix, myometrium and fetal membranes during labour and before the onset of labour, in particular macrophages, neutrophils, T-lymphocytes and B-lymphocytes. Leukocytes are recognized as a major source of cytokines.

#### **4:2. IL-1 $\beta$ .**

IL-1 is a "pro-inflammatory" cytokine. Two isoforms have been identified: IL-1 $\alpha$  and IL-1 $\beta$ . The latter predominates in human tissues. Cells known to express IL-1 $\beta$  include natural killer (NK) cells, macrophages, monocytes, endothelial cells, neutrophils, trophoblasts, T-cells, fibroblasts and smooth muscle cells. IL-1 upregulates the expression of cell adhesion molecules

such as ICAM (intercellular adhesion molecule-1) promoting the adhesion of neutrophils, monocytes, T-cells, and B-cells. In capillary endothelial cells IL-1 induces the secretion of chemokines (e.g. monocyte chemoattractant protein-1, MCP-1). MCP-1 promotes chemotaxis and triggers mononuclear cell integrins, thus aiding mononuclear infiltration into areas of initial inflammation. IL-1 also stimulates release of matrix metallo-proteinases (MMPs) such as collagenase in local fibroblasts resulting in ECM degradation. IL-1 increases the synthesis of prostaglandins. Each of these processes is known to occur in association with parturition.

#### **4:2.1. IL-1 $\beta$ in myometrium.**

In earlier studies it has been shown that in primary culture of myometrial cells IL-1 $\beta$  increases nuclear translocation of NF $\kappa$ B, raises PGHS-2 expression and induces up to an 80-fold increase in PG release (Belt *et al* 1999, Pollard *et al* 1996). These data support the hypothesis that elevated intrauterine levels of IL-1 $\beta$  participate in the onset and maintenance of uterine contractions via increased synthesis of contractile prostaglandins by uterine tissues, including the myometrium. IL-1 $\beta$  message and protein have been demonstrated in myometrial lower segment using RT-PCR (Sehringer *et al* 2000) and ELISA (Winkler *et al* 1998) respectively. A greater production of IL-1 $\beta$  protein was seen in advanced compared with early labour (Winkler *et al* 1998). However, myometrial concentrations of the IL-1 receptor (measured by western blotting) decline as labour progresses (Hatthacote *et al* 1999) so that the role of IL-1 in the process of labour is unclear. In this study our findings demonstrate that IL-1 $\beta$  mRNA levels were significantly greater ( $p < 0.02$ ) in biopsies collected following spontaneous labour compared with non-labouring biopsies. IL-1 $\beta$  immunolocalised to both myocytes and leukocytes in the

myometrium and the density of leukocytes was greater in the myometrial biopsies obtained after the onset of labour. The increased density of IL-1 $\beta$  positive leukocytes observed during labour is probably responsible for the increased IL-1 $\beta$  mRNA expression found in labouring vs non-labouring tissues. These results support the hypothesis that invading leukocytes are a source of IL-1 $\beta$ . It has previously been shown that in the myometrium there is a considerable influx of leukocytes concurrent with the onset of labour at term (Thomson *et al* 1999) and in this study we have shown these cell types to be predominantly T-cells, macrophages and neutrophils. Our results are in agreement with those suggesting that IL-1 $\beta$  may have a role in the fundamental mechanism of parturition, as IL-1 $\beta$  is known to stimulate myometrial contractions both directly and via an increase in prostaglandin production (Hertelendy *et al* 1993, Molnar *et al* 1993).

#### **4:2.2. IL-1 $\beta$ in fetal membranes.**

In term amnion, chorion, decidua and in amnion-derived cells, IL-1 $\beta$  induces PGHS-2 mRNA and PGE<sub>2</sub> release (Mitchell *et al* 2000, Potter *et al* 1999, Brown *et al* 1998, Edwin *et al* 1996, Cole *et al* 1995). IL-1 $\beta$  has previously been demonstrated in fetal membranes, and a range of studies have shown greater concentrations in membranes (Keelan *et al* 1999) and in amniotic fluid (Opsjon *et al* 1993) in samples taken after, compared with those taken prior to the onset of labour. In contrast to other reports, we failed to find IL-1 $\beta$  immunostaining in amnion (Steinborn *et al* 1999a, Menon *et al* 1995) although our results showed an increase in mRNA expression in labouring amnion samples. An explanation of our results is that low levels of IL-1 $\beta$  expression are present in amnion, but that our immunohistochemical technique is insufficiently sensitive to detect it. This study showed IL-1 $\beta$  mRNA was expressed in chorio-decidua before the onset of

labour with no significant change after labour. The reason for this lack of change with labour is obscure but suggest that IL-1 $\beta$  may have a different role in amnion and choriondecidua compared with other uterine tissues. This role could possibly begin before the onset of labour in the fetal membranes where IL-1 may be responsible in part for the upregulation of prostaglandin synthesis within the amnion and choriondecidua. The prostaglandin produced within the decidua may well have a key role in providing the stimulus to the myometrium to contract. The prostaglandin produced in the amnion and chorion could be linked to the disarrangement of the collagen bundles in the fetal membranes which might bring about their weakening and rupture. In this study we have shown that leukocytes which invade the fetal membranes during parturition produce IL-1 $\beta$ . These data are in agreement with other published studies (Steinborn *et al* 1999b, Menon *et al* 1995).

#### **4:2.3. IL-1 $\beta$ in cervix.**

There was little IL-1 $\beta$  staining in cervix, except in leukocytes invading at the onset of labour. In the cervix, leukocytes were sparse prior to the onset of labour, but their density increased significantly in labour. We found a significant increase ( $p < 0.02$ ) in expression of IL-1 $\beta$  mRNA in cervical biopsies taken after the onset of labour compared to those obtained before the onset of labour. Our results indicate that cervical leukocytes could be responsible for the increase in IL-1 $\beta$  observed in cervico-vaginal fluid after the onset of labour (Tanaka *et al* 1998, Steinborn *et al* 1995). The work described here supports earlier data demonstrating that there is an influx of leukocytes in the cervix during the process of cervical ripening and parturition (Bokstrom *et al* 1997, Junqueira *et al* 1980). The acknowledged effects of these leukocytes comprise the breakdown and remodelling of cervical tissue via the release of metalloproteinases,

prostaglandins, cell adhesion molecules and nitric oxide (Ledingham *et al* 2001, 2000, Thomson *et al* 1999). IL-1 $\beta$  together with TNF $\alpha$  have been shown to induce expression of TIMPs and MMPs by human cervical fibroblasts and smooth muscle cells (Watari *et al* 1999). The IL-1 $\beta$  and other pro-inflammatory cytokines released by the leukocytes and other cell types may be a factor in the process of breakdown and remodelling cervical tissue not least by advancing an additional influx of leukocytes.

#### **4:3. IL-6.**

IL-6, like IL-1, is a pro-inflammatory cytokine and it is known to target various cells including T- and B-lymphocytes, hepatocytes, thymocytes, fibroblasts and monocytes. IL-6 has a vital function in most acute inflammatory responses such as activation of complement, opsonization of bacteria, recruitment of neutrophils, elevation of basal body temperature and activation of T- and B-lymphocytes.

##### **4:3.1. IL-6 in myometrium.**

In this study we immunolocalised IL-6 in infiltrating leukocytes in upper and lower segment myometrium. We demonstrated that levels of mRNA were significantly greater ( $p < 0.005$ ) in myometrial tissue obtained after the onset of labour compared with myometrium sampled before the onset of labour. An earlier study has established that term human myometrium releases IL-6 in culture, and both preterm and term myometrium contain IL-6 mRNA (Schringer *et al* 2000). Our study suggests that leukocytes are the key source of this IL-6. Not all CD45 positive cells stained positively for IL-6. Morphological examination of IL-6 and CD45 positive cells suggested that they are neutrophils, however, additional work is necessary to verify this. Since

infiltrating leukocytes are a major source of IL-6, and given that the density of infiltrating leukocytes is significantly increased following the onset of labour, one might conclude labour to be linked with a rise in myometrial IL-6 concentrations.

#### **4:3.2. IL-6 in fetal membranes.**

We demonstrated IL-6 expression in amnion and chorion, in line with published reports (Menon *et al* 1995). The cells expressing IL-6 within the chorion appeared (from their morphology) to include decidual cells, extravillous trophoblast and leukocytes. To confirm that these cells are decidual cells and trophoblasts serial sections of tissue could be immunostained using vimentin and cytokeratin antibodies respectively. Any or all of these cells could be responsible for the increase in IL-6 production observed in fetal membranes and amniotic fluid following the onset of labour (Keelan *et al* 1999, Opsjon *et al* 1993). We have shown that levels of IL-6 mRNA are significantly increased in choriodecidua samples obtained after the onset of labour, when compared with choriodecidua taken before the onset of labour. There was little IL-6 mRNA expression in amnion either in labour or not in labour samples. These data concur with those showing amniochorionic expression only when samples were obtained from infected membranes or were stimulated with by endotoxin (Menon *et al* 1995) whereas amniochorionic IL-6 protein expression has been more consistently identified (Keelan *et al* 1997, Laham *et al* 1996).

#### **4:3.3. IL-6 in cervix**

An elevation in IL-6 concentrations in cervico-vaginal fluid has been observed during labour at term and preterm, and evaluation of cervico-vaginal IL-6 has been suggested as a method of predicting delivery in women in suspected preterm labour (Burrus *et al* 1995). In cervix, we

immunolocalized IL-6 to glandular and surface epithelium and levels of mRNA were significantly greater ( $p < 0.05$ ) in cervix samples obtained after the onset of labour when compared with those samples collected before the onset of labour. In addition we demonstrated a significant increase in leukocytes, mainly T-cells and neutrophils in labouring cervix biopsies. We are not aware of any studies localizing IL-6 within the pregnant cervix. However, our results showing IL-6 localization in superficial and glandular epithelium are in agreement with those found in non-pregnant cervix (Frohm Nilsson *et al* 1999). Once more, infiltrating leukocytes, chiefly T-cells and neutrophils expressed IL-6, and these may be the source of the increased levels of IL-6 protein and mRNA seen in connection with cervical ripening (Sennstrom *et al* 2000).

#### **4:4. IL-8.**

The pro-inflammatory cytokine IL-8 is produced by a wide variety of cells including lymphocytes, fibroblasts, and epithelial and endothelial cells when stimulated by endotoxin, TNF or IL-1. IL-8 is a chemotactic factor that attracts leukocytes, and stimulates adhesion molecule expression and neutrophil activity.

##### **4:4.1. IL-8 in myometrium.**

Many previous studies have established the changes in IL-8 concentrations during pregnancy. IL-8 production (measured by ELISA) is greater in labouring versus non-labouring term myometrium, and correlates with increasing cervical dilation (Winkler *et al* 1998, Osmers *et al* 1995). IL-8 mRNA was identified in labouring but not in non-labouring myometrial samples using RT-PCR (Segringer *et al* 2000). The effects of IL-8 within reproductive tissues depend not

only on IL-8 concentration but also on the concentration of its receptor. IL-8 receptor mRNA declines in the myometrium in association with labour at term, possibly as a result of increased IL-8 concentrations (Hatthacote *et al* 1999). In this study we have confirmed the presence of IL-8 mRNA in labouring myometrium and its absence in non-labouring myometrium. We have shown that cells within the myometrium which express IL-8 also express CD45 and that these cells are predominantly T-cells, macrophages and neutrophils. IL-8 expression was not identified in any other cell types. Macrophages, neutrophils and to a lesser extent T- and B- lymphocytes are known to infiltrate the myometrium in great numbers at the time of parturition (Thomson *et al* 1999). The data presented in this study suggest that these invading leukocytes are the only source of the major increase in myometrial IL-8 production occurring at the time of labour.

#### **4:4.2. IL-8 in fetal membranes.**

Whether there is an increase in production of IL-8 from fetal membranes in association with labour is more controversial. An increase has been demonstrated in some studies (Keelan *et al* 1999) but no change in other studies (Laham *et al* 1999, Osmers *et al* 1995). Type I and II receptors have been identified in fetal membranes and myometrium at term before the onset of labour and staining was more intense in amnion obtained after the onset of labour when compared with that obtained before (El Maradny *et al* 1996). A previous study has also shown IL-8 receptor expression in decidua is induced by mechanical stretch (Maehara *et al* 1996). In none of these studies was the cell type responsible for IL-8 production identified. In this study we have demonstrated that ratios of IL-8 mRNA to GAPDH mRNA are significantly higher in amnion ( $p < 0.01$ ) and choridecidua ( $p < 0.005$ ) obtained after the onset of labour when compared with samples taken pre-labour. We have also shown that cells within fetal membranes which

express IL-8 also express CD45. IL-8 expression was not identified in any other cell types. As previously discussed the effects of IL-8 within uterine tissues depend not only on IL-8 concentration but also on the concentration of its receptor, while IL-8 receptor mRNA declines in the myometrium in association with labour at term, in contrast, amniotic fluid concentrations of IL-8 receptor increase in association with preterm labour in the absence of infection (Arntzen *et al* 1998). The net effects of IL-8 in parturition may change therefore within different uterine tissues.

#### **4:4.3. IL-8 in cervix.**

The function of IL-8 in cervical ripening is well recognized. In animal studies IL-8 induces cervical remodelling and dilatation of the cervix (Barclay *et al* 1993). Collagen content, water content, neutrophil density, elastase and collagenase activity of rabbit cervixes are increased by IL-8 (Winkler *et al* 1999b). In a previous study it has been suggested that IL-8 is the mediator of cervical ripening at least in humans (Sennstrom *et al* 1997). It has also been shown that there is a corresponding rise in IL-8 mRNA and protein synthesis with cervical ripening (Sennstrom *et al* 2000). In this study we demonstrated a significantly greater concentration of mRNA levels in cervical biopsies obtained after the onset of labour compared with biopsies sampled before the onset of labour. We immunolocalized IL-8 to squamous and glandular epithelial cells, and to stromal cells – these data are in agreement with the previously published data (Sennstrom *et al* 2000). Additionally in our study, we have shown a marked increase in density of leukocytes chiefly T-cells and neutrophils in labouring cervical samples. These results confirm data from previous studies showing that the cervix is infiltrated by leukocytes during cervical ripening and parturition (Bokstrom *et al* 1997, Junqueira *et al* 1980). The accepted effects of these leukocytes

comprise breakdown and remodelling of cervical tissue through release of prostaglandins, metalloproteinases, cell adhesion molecules and nitric oxide (Ledingham *et al* 2000, Thomson *et al* 1999). Identification of these leukocytes by immunocytochemistry in serial sections using CD45 and anti-IL-8 antibodies has shown that IL-8 is also localized to these infiltrating inflammatory cells. The pro-inflammatory cytokine IL-8 released by these leukocytes may contribute to the process of tissue breakdown and remodelling. Further work is necessary to determine the comparative input of native cervical cell types and invading leukocytes to the increased levels of IL-8 seen in connection with parturition.

#### **4:5. TNF $\alpha$ .**

TNF $\alpha$  is known to be secreted by a variety of leukocytes, principally macrophages. TNF $\alpha$  secretion has also been demonstrated in some cell lines, including smooth muscle cells and fibroblasts. TNF $\alpha$  and IL-1 are compatible cytokines mediating many of the same functions during parturition.

##### **4:5.1. TNF $\alpha$ in myometrium.**

Within the myometrium, we immunolocalised TNF $\alpha$  to a small subpopulation of leukocytes only. The morphology of these leukocytes suggests that they are macrophages, however, immunostaining in serial sections with a macrophage marker is necessary to confirm this hypothesis. Other studies on human pregnant myometrium have shown either low levels of TNF $\alpha$  using enzyme immunoassay (Sehringer *et al* 2000) or levels below the limit of detection using ELISA (Osmers *et al* 1995). Utilizing RT-PCR small amounts of TNF $\alpha$  mRNA were identified in myometrium from 2 out of 5 women in labour (Sehringer *et al* 2000). TNF $\alpha$  has

also been shown to induce the expression of extracellular remodelling enzymes MMP-1 and MMP-3 in myometrium (Roh *et al* 2000). Both TNF $\alpha$  and IL-1 $\beta$  potentially play a role in labour since both IL-1 $\beta$  and TNF $\alpha$  stimulate uterine contractions. These cytokines stimulate arachidonic acid release and prostaglandin production in human myometrial cells thus stimulating myometrial contractions (Molnar *et al* 1993). However the data showing the expression of TNF $\alpha$  in only a subpopulation of lymphocytes within the myometrium and that TNF $\alpha$  mRNA expression was below the limits of detection of our assay means that the functional role of TNF $\alpha$  is uncertain.

#### **4:5.2. TNF $\alpha$ in fetal membranes.**

In other studies it has been shown that TNF- $\alpha$  is released from fetal membranes and is elevated in both vaginal secretions and amniotic fluid in labour (Fortunato *et al* 1996, Steinborn *et al* 1995, Opsjon *et al* 1993). A previous study has immunolocalised TNF- $\alpha$  to CD11b positive cells (a leukocyte subpopulation) and to decidual cells in membranes (Steinborn *et al* 1999). This is in agreement with the results of this study. In this study TNF $\alpha$  mRNA expression was below the limits of detection of our assay. TNF- $\alpha$  also stimulates matrix metalloproteinase production in human fetal membranes (Kent *et al* 1993, So *et al* 1992). A study demonstrating an association between polymorphism of the promotor of the TNF- $\alpha$  gene with increased risk of preterm premature rupture of the membranes (Roberts *et al* 1999) gives support for a role for TNF in tissue remodelling and rupture within the fetal membranes.

#### 4:5.3. TNF $\alpha$ in cervix.

In cervical smooth muscle cells TNF induces the expression of matrix metalloproteinase mRNA (Watari *et al* 1999). In this study within the cervix, we localized TNF- $\alpha$  to infiltrating leukocytes and cervical epithelial cells. We are not aware of any other data localizing TNF- $\alpha$  production within the pregnant cervix. Once more, in our study only a subpopulation of leukocytes expressed TNF- $\alpha$ , these leukocytes had the morphology of macrophages, however immunostaining in serial sections with a macrophage marker is necessary to confirm their surface antigen expression. These results lend support for a role of TNF $\alpha$  in tissue remodelling that occurs during parturition and immediately afterwards (Roh *et al* 2000).

#### 4:6. Leukocyte density in reproductive tissue after the onset of labour.

In this study 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 observed an influx of inflammatory cells in the myometrium and cervix only, and not in the fetal membranes or decidua in association with parturition.

In the cervix, leukocytes were sparse before the onset of labour, but their density was increased significantly in labour together with increased cervical production of pro-inflammatory cytokines. Indeed, there was a significant correlation between mRNA expression of each of the cytokines IL-1 $\beta$ , IL-6 and IL-8 and total leukocyte density within the cervix, 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]. As previously mentioned, we localised each of the

pro-inflammatory cytokines to the leukocytes within the cervix suggesting that infiltrating leukocytes are a major source of the increase in these mediators during parturition. The work described here confirms previous data showing that the cervix is invaded by leukocytes during the process of cervical ripening and parturition (Bokstrom *et al* 1997, Junqueira *et al* 1980). The recognized effects of these leukocytes include breakdown and remodelling of cervical tissue via release of matrix metalloproteinases, prostaglandins, cell adhesion molecules and nitric oxide (Ledingham *et al* 2001, 2000, Thomson *et al* 1999). The pro-inflammatory cytokines released by the leukocytes and other cell types may also be a factor in this process, by promoting further leukocyte invasion. The work described here is at variance with earlier reports as to the exact timing of the leukocytic invasion (Bokstrom *et al.*, 1997). In this 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 comparable (when corrected for the area examined) in both his 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 characterize active labour, whereas we used strict inclusion criteria recruiting only those women in spontaneous labour with cervical dilation of between four to eight 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 connection with labour might have been masked. If the non-labouring women in our study were further from the onset of spontaneous labour (with a less ripe cervix) than the Bokstrom subjects, the rise in leukocyte count which we observed could have arisen 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 a rise 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. Again, as previously referred to, data in this study suggests that the invading leukocytes make a significant contribution to pro-inflammatory cytokine production. As mentioned earlier in this discussion one of the cytokines produced, IL-1 $\beta$ , 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.

**4:7. Limitations of the study.**

As levels of TNF $\alpha$  mRNA were below the limits of detection in our assay, northern analysis may not be a sensitive enough assay to employ. The use of real time PCR may be a more appropriate analysis to use. The upregulation of mRNA does not necessarily mean protein synthesis is upregulated, to determine if there is an increase in protein synthesis in tissue obtained after the onset of labour it would be necessary to carry out western blotting on protein samples prepared from tissue samples. Using immunocytochemistry we demonstrated subpopulations of leukocytes in uterine tissues, to evaluate exactly which cytokines these different leukocytes express, fluorescence activated cell sorting (FACS) analysis may be employed. To determine minor increases in levels of mRNA between samples obtained before and after the onset of labour a larger sample size would have to be investigated.

**4:8. Summary.**

As far as we can ascertain this is the first time that peripartum pro-inflammatory events have been evaluated in each of the major tissue types in the uterus using the same experimental protocol. We have immunolocalized production and quantified mRNA of each of the inflammatory mediators IL-1 $\beta$ , IL-6, IL-8 and TNF $\alpha$  in the myometrium, fetal membranes and cervix before and after labour. We have shown that the onset of parturition is linked with a rise in pro-inflammatory cytokines in myometrium, cervix, amnion and chorio-decidua. In contrast, we observed an influx of inflammatory cells in the myometrium and cervix only, and not in the fetal membranes or decidua in association with parturition. We have shown that the leukocytes which invade these tissues are rich sources of inflammatory cytokines. The function(s) of these proinflammatory cytokines in the process of parturition remains unclear. Believing that if these

pro-inflammatory cytokines do have a particular function, rather than being the by-product of another physiological event, possible effects include stimulation of uterine activity, either directly or via an increase in prostaglandin production, attraction of leukocytes and tissue remodelling.

Cytokines may be responsible for mediating the attraction of leukocytes which takes place at the time of parturition and therefore mediate the leukocyte infiltration, either directly (in the case of IL-8) or via upregulation of cell adhesion molecules (IL-1 and TNF- $\alpha$ ). We and others have shown a substantial upregulation of adhesion molecule expression at the onset of labour (Ledingham *et al* 2001). Additionally amniotic fluid IL-1, IL-6 and TNF- $\alpha$  concentrations correlate with the level of leukocyte infiltration in the placenta and membranes (Halgunset *et al* 1994). These data give rise to the possibility that a positive feedback mechanism exists, whereby pro-inflammatory cytokines attract leukocytes into reproductive tissues at the time of parturition. These leukocytes then produce pro-inflammatory cytokines, which attract further leukocytes into the tissues.

Pro-inflammatory cytokines may be significant in tissue remodelling. It has been demonstrated that IL-1 and TNF- $\alpha$  upregulate production of matrix metalloproteinase-9 in human myometrial smooth muscle cells, and might as a result participate in the tissue remodelling which occurs during parturition and immediately thereafter (Roh *et al* 2000). TNF- $\alpha$  also stimulates matrix metalloproteinase production in human fetal membranes (Kent *et al* 1993, So *et al* 1992).

There is confirmation for the hypothesis that the cytokines we have studied stimulate indirectly uterine contractions. Both IL-1 and TNF- $\alpha$  stimulate arachidonic acid release and prostaglandin production in human myometrial cells consequently stimulating myometrial contractions and

ripening of the uterine cervix (Hertelendy *et al* 1993, Molnar *et al* 1993). Furthermore, IL-1 potentiates oxytocin induced myometrial contractions in an *in vitro* system (Molnar *et al* 1993). The myometrium and fetal membranes may have complementary roles during the process of labour – the activation of parturition could be conveyed from the fetal membranes, perhaps responding to signals received from the fetus (Challis *et al* 2000).

The data presented in this study supports the hypothesis that parturition is an inflammatory process.

#### **4:9. Future studies.**

Further work, utilizing laser scanning cytometry (LSC) or fluorescence activated cell sorting (FACS) following cell extraction by enzyme treatment, might quantify the comparative input of inflammatory cells and other cell types to cytokine production around the time of parturition. FACS analysis would identify precisely which cells are expressing each of the cytokines and their density which we have explored in this study and confirm our results. Assuming that this work corroborates the data described in this study, we expect that strategies such as anti-TNF therapy, which has been used successfully in humans to treat the chronic autoimmune inflammatory diseases Crohn's disease (van Dullemen *et al* 1995) and rheumatoid arthritis (Elliot *et al* 1994), and other anti-cytokine biologicals designed to reduce the inflammatory infiltrate, or inhibit cytokine production in cells by blocking the downstream effects of the many pro-inflammatory cytokines may provide novel, therapeutic and effective treatment of preterm labour.

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**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	<i>P</i>	Before labour	After labour	<i>P</i>
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-1 $\beta$ , 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- $\alpha$  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.

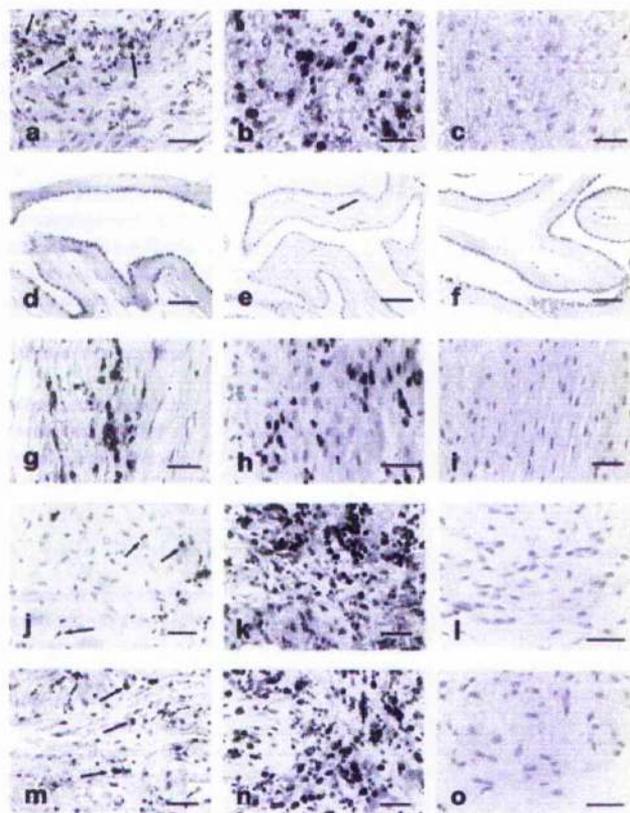
Within cervical tissue, there was a significant correlation between mRNA expression of each of IL-1 $\beta$ , IL-6 and IL-8 and total leukocyte density, after adjusting for the presence or absence of labour [partial correlation coefficients of 0.94 ( $P < 0.02$ ), 0.94 ( $P < 0.02$ ) and 0.92 ( $P <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

### Amnion and chorio-decidua

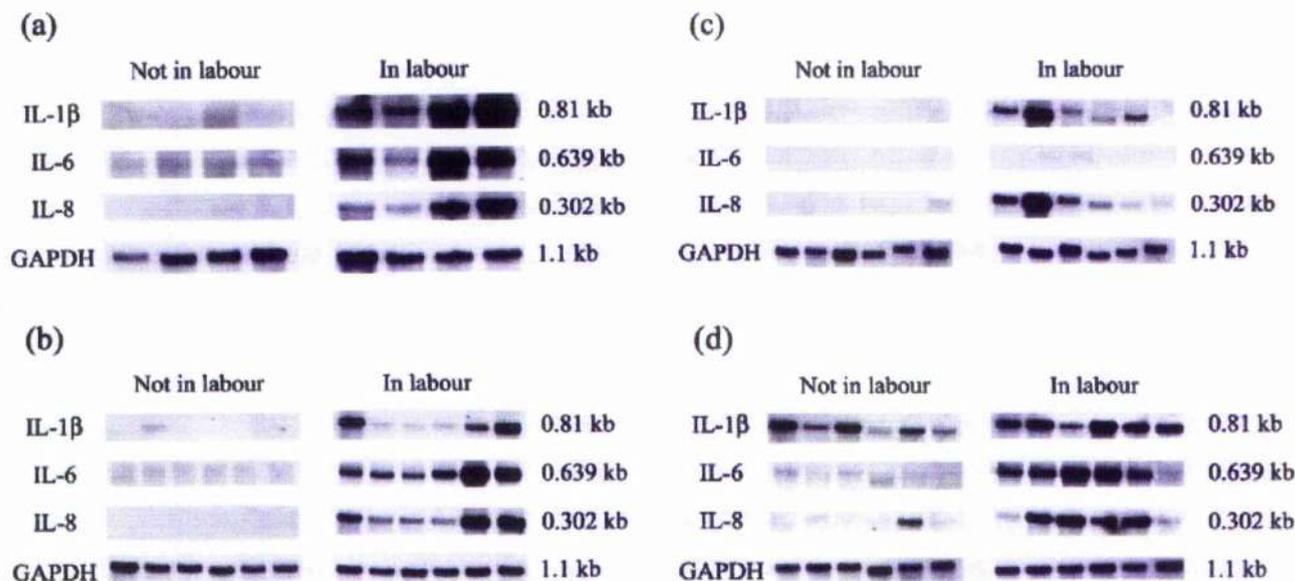
As with the cervix, we were able to identify inflammatory cells in the amnion and chorio-decidua (Figure 1). The median density of leukocytes, macrophages, neutrophils, T-lymphocytes and B-lymphocytes was 1 or <1 per high powered field in the amnion and chorion with no significant differences after labour 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-1 $\beta$  ( $P < 0.02$ ) 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-1 $\beta$  mRNA was significantly greater in chorio-decidua (Figure 2d) following spontaneous labour. TNF- $\alpha$  mRNA expression was detected neither by Northern analysis nor by PCR.

## 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 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  $\mu$ m. The light grey background is haematoxylin counterstain.



**Figure 2.** Northern blot hybridization of total RNA (10  $\mu$ g 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-1 $\beta$ , 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-1 $\beta$ , IL-6 and IL-8 mRNA was up-regulated following the onset of labour. In amnion (c), the expression of IL-1 $\beta$  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-1 $\beta$  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- $\alpha$  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-1 $\beta$ , 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-1 $\beta$  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-1 $\beta$  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 significantly 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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Submitted on July 5, 2002; accepted on October 2, 2002