

Preterm Cervical Ripening in humans

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Abstract

Preterm birth (PTB) is the leading cause of neonatal mortality and morbidity. Despite the current treatment procedures, the incidence of PTB has not changed in the past thirty years. Incomplete understanding of the biological and pathophysiological mechanisms underlying preterm delivery is the major obstacle to prevent PTB. Cervical ripening is necessary for vaginal delivery and understanding of preterm cervical ripening is required for developing new treatment strategies. Several important substances such as HMGB1 and its receptors, CRH and its receptors and numerous cytokines are localized in the cervix and undergo distinct changes in labour. Other important molecules, such as CRH, CRH-BP, CRH-R1, CRH-R2, HMGB1, TLR2, TLR4, IL-10, IL-12, are localized in the cervical epithelium, also indicating their role in the process of cervical ripening during labour. Furthermore, CRH stimulates IL-8 secretion from both preterm and term cervical fibroblasts. Recent studies from our group show that major inflammatory changes occur in the cervix at labour irrespective of gestational age. This indicates that cervical ripening at both term and preterm is an inflammatory process even if no infection is present. However, preterm cervical ripening still entails some differences from term cervical ripening, for example in the down-regulation of mRNA expression of Toll-like receptors (TLR-2 and TLR-4) and IL-12, higher levels of IL-10 in cervical epithelium, and presents different secretion patterns of cervical fibroblasts. Moreover, preterm cervical ripening, like preterm delivery itself, is a multifactorial disorder with pathways which are partly different from those involved in PPRM and infected preterm labour.

Key words: Cervical ripening, cytokines, CRH, cervical fibroblasts, HMGB1, preterm labour.

Introduction

Preterm birth can be divided into elective deliveries due to maternal or foetal indications, and spontaneous preterm birth with or without preterm premature rupture of membranes (PPROM) (Goldenberg et al., 2008). Multiple causes i.e. infection, utero-placental ischemia or haemorrhage, stress, endocrine factors, immunologically mediated processes may be associated with preterm birth (Challis et al., 2009) (Fig. 1).

Current tocolytic therapies have not reduced the incidence of preterm birth. Thus there is a need for new strategies for treatment and prevention of preterm birth. The current treatment strategies focus upon tocolytic agents and upon diminishing the uterine contractility. However, a preterm vaginal birth first requires cervical ripening after which

myometrial contractions follow. Indeed, even painfully strong contractions in combination with stiff and closed cervix do not result in preterm delivery. In order to develop new strategies to diminish the preterm birth rate, it is therefore of major importance to improve our understanding of the physiology of preterm cervical ripening.

Composition of Cervix uteri

Danforth et al. (1947) stated that the human cervix consists mainly of fibrous connective tissue. The non-pregnant cervix is composed of 85% extracellular matrix (ECM) and 6-10% of muscle fibers (Rorie and Newton, 1967). The cervical ECM consists of collagens, proteoglycans, hyaluronan, and glycoproteins. Collagen I and collagen III are the major types and the dominating proteoglycan in

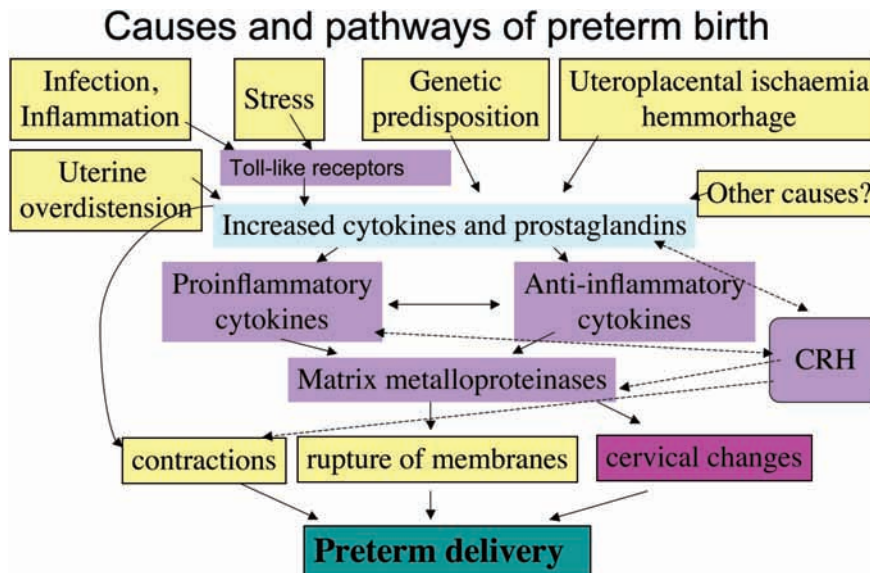


Fig. 1. — Causes and pathways of preterm birth

non-pregnant cervix uteri is decorin. Fibromodulin and biglycan, and also large proteoglycans such as versican and heparan sulfate proteoglycan are present (Uldbjerg et al., 1983b; Norman et al., 1991; Westergren-Thorsson et al., 1998).

Cervical ripening at term

Cervical softening during pregnancy is characterized by a gradual decrease in the collagen concentration. Simultaneously the collagen extractability increases, suggesting changes in the organization of collagen fibrils (Uldbjerg et al., 1983a; Granstrom et al., 1989). The duration of cervical dilatation during labour correlates well with the collagen concentration and its solubility (Ekman et al., 1986). Immediately after vaginal delivery at term the mRNA levels for collagen I and III decrease by up to 60% compared to those in the non-pregnant cervix, but during involution (2-4 days after delivery) the levels are increased 2.5-fold and 3.5-fold respectively (Westergren-Thorsson et al., 1998).

The decorin concentration is decreasing with 50% until final ripening while that of versican, biglycan and the heparan sulfate proteoglycans increases (Norman et al. 1993; Westergren-Thorsson et al. 1998). Versican can attract water and bind hyaluronan (Wu et al., 2005), resulting in disintegration of the collagen bundles and a change in the physical properties to produce a soft and elastic tissue, thus facilitating cervical dilatation.

Gonadal steroids

Estrogen, progesterone and insulin-like growth factor-I (IGF-I) are involved in cervical ripening

(Stjernholm et al., 1996; Stjernholm et al., 1997; Wang et al., 2001). ER α mRNA decreases in the ripe cervix at delivery, while ER β mRNA levels are increased in the term pregnant cervix not in labour (Wang et al., 2001). The ER β antigen is also co-localized with leukocyte markers in cervix (Stygar et al., 2001).

Cervical ripening – an inflammatory reaction

Further, the process of cervical ripening at labour can be regarded as an inflammatory reaction since the levels of IL-6, IL-8 increase at term labour (Sennstrom et al., 2000). This process is also associated with cervical leukocyte invasion (Young et al., 2002; Osman et al., 2003).

Cytokines recruit activated cells, which in turn secrete degradative enzymes such as matrix metalloproteinases (MMPs). Thus, increased levels of MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 have been observed during pregnancy and at the final cervical ripening (Stygar et al., 2002; Sennstrom et al., 2003).

Fibroblasts

Fibroblasts play a crucial role in the remodelling of the extracellular matrix (Larsen et al. 2006). Activated fibroblasts produce ECM components, cytokines and matrix metalloproteinases (Malmstrom et al., 2007; Akerud et al., 2008). Cervical fibroblast cultures established from the biopsies from non-pregnant women, term pregnant women at caesarean section before the onset of labour or parturient women, present different and stable phenotypes (Malmstrom et al., 2007). There is a decrease in pro-

teoglycan secretion and an increase in IL-6, IL-8, MMP-1, MMP-3 production in the cultures from parturient donors (Malmstrom et al., 2007; Akerud et al., 2008).

Preterm cervical ripening

Prostaglandins

Prostaglandins, synthesized in foetal membranes and deciduas play an important role in parturition and cervical ripening. Local application of prostaglandin-E₂ (PGE₂) has become a routine treatment in inducing cervical ripening and labour both at term and at preterm (Ekman et al., 1983; Abelin Tornblom et al., 2002). Cervical ripening at preterm as well as at term is associated with decreased degradation of prostaglandins (Tornblom et al., 2004).

NO

Nitric oxide (NO) has been suggested as an active mediator in cervical ripening (Chwalisz and Garfield, 1998), and NO-donors induce ripening of the human cervix (Thomson et al., 1997). The expression of nitric oxide synthase (NOS) isoforms increases in the cervix in late pregnancy and parturition (Ledingham et al., 2000). Further, preterm labour is associated with higher mRNA expression of NOS isoforms in the cervix (Tornblom et al., 2005b). Cervical fluid nitric oxide metabolite (NO_x) levels rise during labour, nitric oxide donor administration or cervical manipulation and are significantly related to cervical ripening. (Vaisanen-Tommiska et al., 2003).

Corticotropin-releasing hormone, CRH

CRH, also termed corticotropin-releasing factor (CRF) is the principal regulator of the hypothalamic-pituitary-adrenal (HPA) axis (Hillhouse and Grammatopoulos 2006).

CRH, CRH-BP, CRH-R1 and CRH-R2 have been identified at both mRNA and protein level in human placenta, deciduas, foetal membranes, endometrium and myometrium (Petraglia et al., 1992; Petraglia et al., 1993; Hillhouse and Grammatopoulos, 2002; Sehringer et al., 2004). Furthermore, CRH increases MMP-9 protein secretion by cultured cells from placenta and foetal membranes (Li and Challis, 2005). In addition, several studies have shown that CRH can stimulate the production of cytokines in different types of cells (Wang et al., 2007).

Klimaviciute et al (2006) found differences in CRH-BP, CRH-R1 and CRH-R2 mRNA expression in cervical tissue and myometrium. These changes

seem to be related to pregnancy and labour but not to gestational age. CRH-BP, CRH-R1, CRH-R2 are down regulated during pregnancy. CRH-R2 in cervix and myometrium and CRH-BP in cervix are even more down regulated during labour. With immunohistochemistry CRH was localized in cervical epithelium with the highest concentration at term, while CRH-BP is decreased at labour, which shows possible involvement of CRH in cervical ripening (Klimaviciute et al., 2006).

In vitro CRH stimulates IL-8 production in the cultures of preterm and term cervical fibroblasts, but doesn't seem to have effect on the secretion of MMP-1 and MMP-3 (Dubicke et al., 2008) (Fig. 2).

High-mobility group box protein 1 (HMGB1)

HMGB1 is expressed by almost all cells (Bianchi and Manfredi, 2007). In 1999 it was discovered that activated macrophages secrete HMGB1 as a delayed mediator of inflammation (Wang et al., 1999). HMGB1 has also important extracellular cytokine-like functions mediating the late response to infection, injury and inflammation (Lotze and Tracey, 2005). HMGB1 induces NF- κ B activation (Lotze and Tracey, 2005) and stimulates pro-inflammatory cytokine synthesis (Andersson et al., 2000).

Human term placenta expresses HMGB1. Labour does not influence its placental expression, although a tendency towards higher expression of extranuclear HMGB1 in placentas with preeclampsia has been observed (Holmlund et al., 2007). HMGB1 is expressed in human foetal membranes at term pregnancy (Ticconi et al., 2007).

A receptor for advanced glycation end-products (RAGE) and Toll-like receptor 2 (TLR2) and TLR4 are involved in HMGB1-mediated signalling (Bianchi and Manfredi, 2007).

Receptor for advanced glycation end-products (RAGE)

The receptor for HMGB1 is RAGE, a multiligand receptor of the immunoglobulin family. The soluble form of RAGE (sRAGE) receptor is considered to act as a regulator/inhibitor of HMGB1 action (Lotze and Tracey, 2005). RAGE is expressed in trophoblasts of first-trimester human chorionic villi from healthy women (Konishi et al., 2004). Human term placenta expresses RAGE, but labour does not influence this expression (Holmlund et al., 2007). RAGE staining is especially increased in the vasculature of myometrium in women with preeclampsia (Cooke et al., 2003). There are contradictory results concerning intra-amniotic infection/inflammation

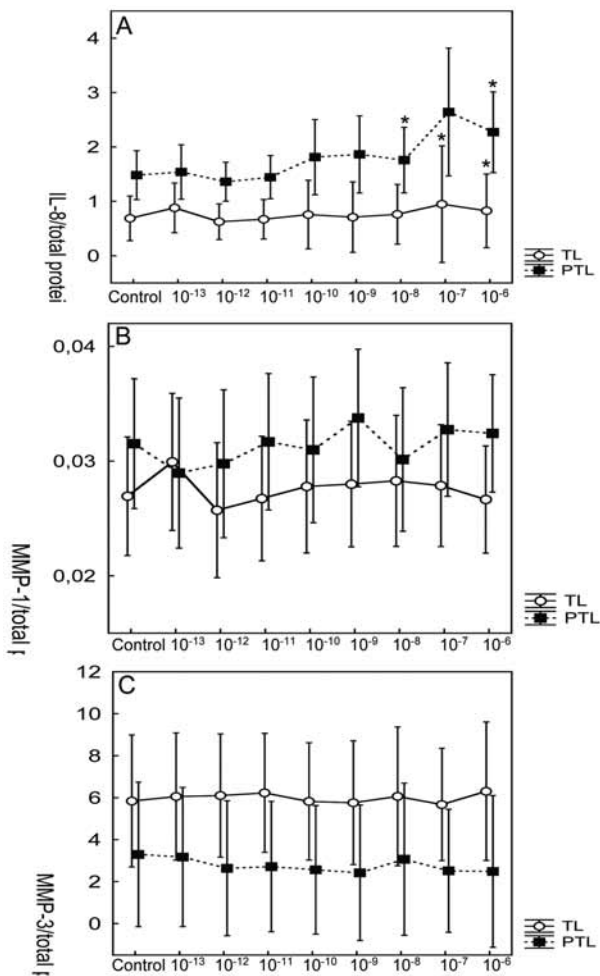


Fig. 2. — Effect of CRH on secretion of IL-8 (A), MMP-1 (B) and MMP-3 (C) by preterm (PTL) and term (TL) cervical fibroblasts. Different concentrations (M) of CRH indicated on x-axis. Data presented as mean \pm SD. * $p < 0.05$ (compared with control). The secretion of IL-8 and MMP-1 was significantly higher in preterm cervical fibroblasts ($p < 0.001$ and $p < 0.05$, respectively). MMP-3 secretion was significantly higher in term cervical fibroblasts ($p < 0.001$).

and amniotic fluid concentrations of sRAGE (Buhimschi et al., 2007; Romero et al., 2008). Women with threatening preterm birth had significantly higher serum sRAGE concentrations than healthy pregnant women.

Toll-like receptors (TLRs)

These receptors are expressed on different cell types belonging to the innate, adaptive immune system and non-immunological cells. TLR2 and TLR4 were the first TLRs to be demonstrated at the protein level in human term placenta (Holmlund et al., 2002). Expression of all ten TLRs has been described in human placenta and studied in pregnancy and in labour (Patni et al., 2007). Greater mRNA expression is seen of all TLR genes in the placenta during labour (Patni et al., 2009). TLRs in the cervix have

been less extensively studied. In pregnant mice, increased mRNA expression of TLR2, TLR3 and TLR4 is seen (Gonzalez et al., 2007). TLRs 1 to 6 mRNA and the protein of TLR2 and TLR4 have been found in human non-pregnant cervix (Pioli et al., 2004). Up-regulation of mRNA expression of TLR2 and TLR4, but down-regulation of TLR3 and TLR5, were observed in the cervix during labour with microarray analysis. The down-regulation of TLR3 and TLR5, but not up-regulation of TLR2 and TLR4 was confirmed with real-time RT-PCR (Has-san et al., 2006).

Dubicke et al found HMGB1, RAGE, TLR2 and TLR4 to be localized and to have an mRNA expression in the human cervix (Dubicke et al., 2010a) There was more extranuclear HMGB1 in the cervical epithelium and stroma in the preterm and term labour groups (Fig. 3). Extranuclear expression of HMGB1 during labour suggests a possible role of HMGB1 during the process of cervical ripening. There was a lower staining and tendency to lower mRNA expression for HMGB1 in the labouring groups. mRNA for RAGE was down regulated in the labouring groups, but there was higher concentration of soluble RAGE in labour. There was an up-regulation of TLR2 mRNA expression in labour. On the contrary, there were lower protein levels of TLR2 and TLR4 in labour (Fig. 4). The preterm group showed lower mRNA expression for TLR2 and TLR4 than term labour group (Fig. 4, C-D). Changes in mRNA expression of HMGB1, TLR2 and TLR4 in preterm labour, point towards possible differences in the mechanism of cervical ripening at preterm and term (Dubicke et al., 2010a).

Cytokines

Tornblom et al found in cervical biopsies obtained at preterm and term pregnancies the protein concentrations of IL-8, IL-6, and MCP-1 to be significantly increased during labour compared to non-labouring groups, whereas no changes were observed for RANTES and TNF- α . The mRNA levels of representative cytokines such as IL-8 and MCP-1 increased significantly whereas RANTES mRNA expression remained unchanged. WBC and CRP were significantly higher in the labouring groups as compared to groups not in labour. For neither of the analysed cytokines, WBC or CRP levels were there any changes between preterm and term groups (Tornblom et al., 2005a).

The role of IL-12 and IL-18 during pregnancy and parturition has attracted interest recently. IL-12 and IL-18 are important in regulating natural killer cell activities in early pregnancy, and are considered important for reproductive success. Higher IL-12 levels

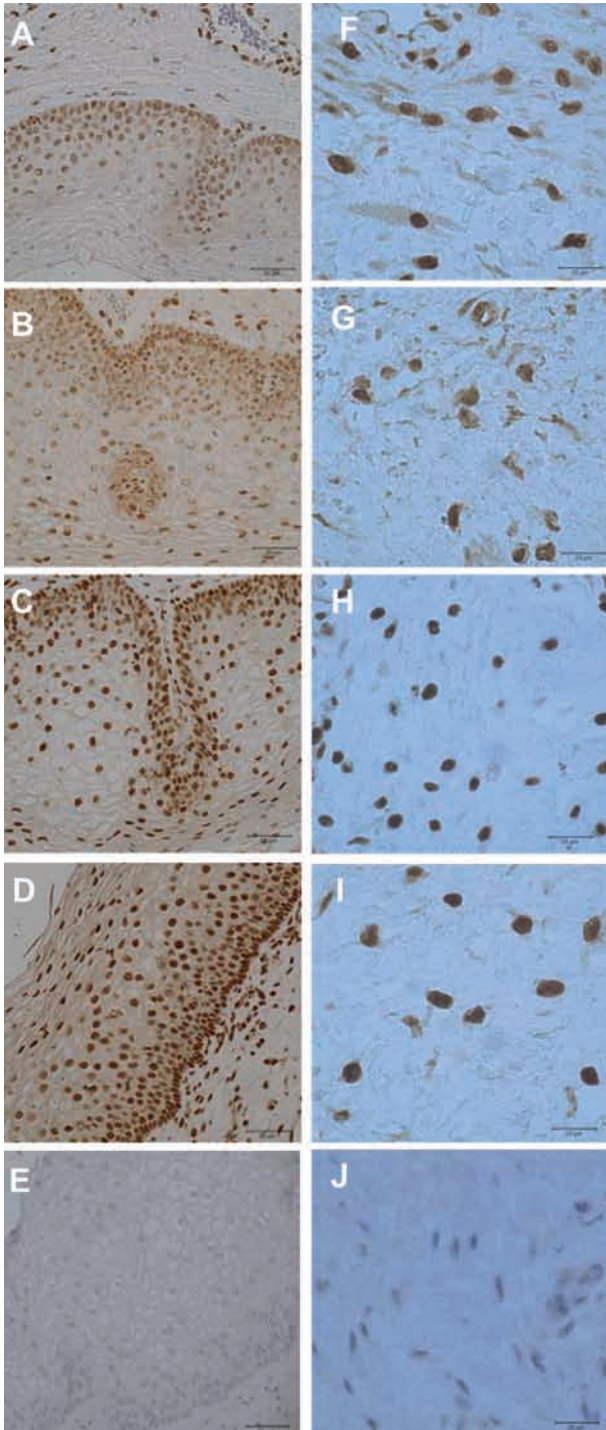


Fig. 3. — The immunohistochemical staining of HMGB1 in the squamous epithelium (column on the left) and in the stroma (column on the right) in the cervical tissue.

HMGB1 in the cervical epithelium at preterm labour (A), term labour (B), term not in labour (C) and non-pregnant state (D). Negative control (E). The magnification is $\times 400$. The mark is $50 \mu\text{m}$.

HMGB1 in the stroma at preterm labour (F), term labour (G), term not in labour (H) and non-pregnant state (I). Negative control (J). The magnification is $\times 1000$. The mark is $20 \mu\text{m}$.

in mid-pregnancy are associated with preterm delivery with chorioamnionitis before 35 weeks of gestation (Gargano et al., 2008). Patients having low IL-18 and high IL-12 had a twofold-increased risk

of delivering before 34 weeks of gestation (Ekelund et al., 2008). When infection is present in preterm labour, higher levels of IL-18 are found in amniotic fluid (Jacobsson et al., 2003). In animal studies, the frequency of foetal loss was significantly higher in IL-18 knock-out mice and in mice receiving IL-18 binding protein than in wild-type controls. IL-18 knock-out mice also present with elevated IL-12 expression in uterine tissues (Wang et al., 2006).

IL-10 is the most extensively studied of the anti-inflammatory cytokines. It decreases the production of pro-inflammatory cytokines such as IL-8, IL-6, TNF- α , IL-1 β (Fortunato et al., 1998; Sato et al., 2003), matrix metalloproteinases (Fortunato et al., 2001) and prostaglandin E₂ (Brown et al., 2000) in LPS-stimulated foetal membranes. The ratio of IL-10/IL-8 decreases in cervical secretions with advancing gestational age (Mondestin-Sorrentino et al., 2007). IL-10 was significantly reduced in placental tissues in chorioamnionitis-associated preterm labour as well as in term labour, compared with second-trimester normal pregnancy samples obtained from elective terminations (Hanna et al., 2006). However, patients who delivered preterm without intra-amniotic infection, had a significantly higher median amniotic fluid IL-10 concentration than those who delivered at term (Gotsch et al., 2008).

IL-4 and IL-13 have been less studied in pregnancy and labour. IL-4 is higher in cervical secretions in women with normal pregnancies not in labour compared to women with preterm labour (Hollier et al., 2004). Higher anti-inflammatory/pro-inflammatory cytokine ratio in cervical secretions during early pregnancy is associated with a higher risk of subsequent spontaneous preterm birth (Simhan and Krohn, 2009). In addition, IL-4, IL-10 and IL-13 gene polymorphism is associated with preterm delivery (Annells et al., 2004; Kerk et al., 2006; Heinzmann et al., 2009).

Dubicke et al. (2010b) reported an up-regulation of mRNA of IL-10, IL-1 α , IL-1 β , but down-regulation of IL-12 and IL-18 in the cervix of the labouring groups compared to the non-labouring group irrespective of gestational age. IL-4 mRNA was detected more frequently in the preterm than in the term labour group. IL-13 was detected more frequently in the labouring groups. IL-12 mRNA expression was lower in the preterm labour group than in the term labour group. The protein levels of IL-10 remained the same in all the groups, IL-4 and IL-12 decreased, while IL-18 increased in the labouring groups. IL-4 protein levels were significantly higher in the preterm subgroup with bacterial infection compared to non-infected group. IL-10 had higher expression in squamous epithelium at preterm labour than at term (Fig. 5) (Dubicke et al., 2010b).

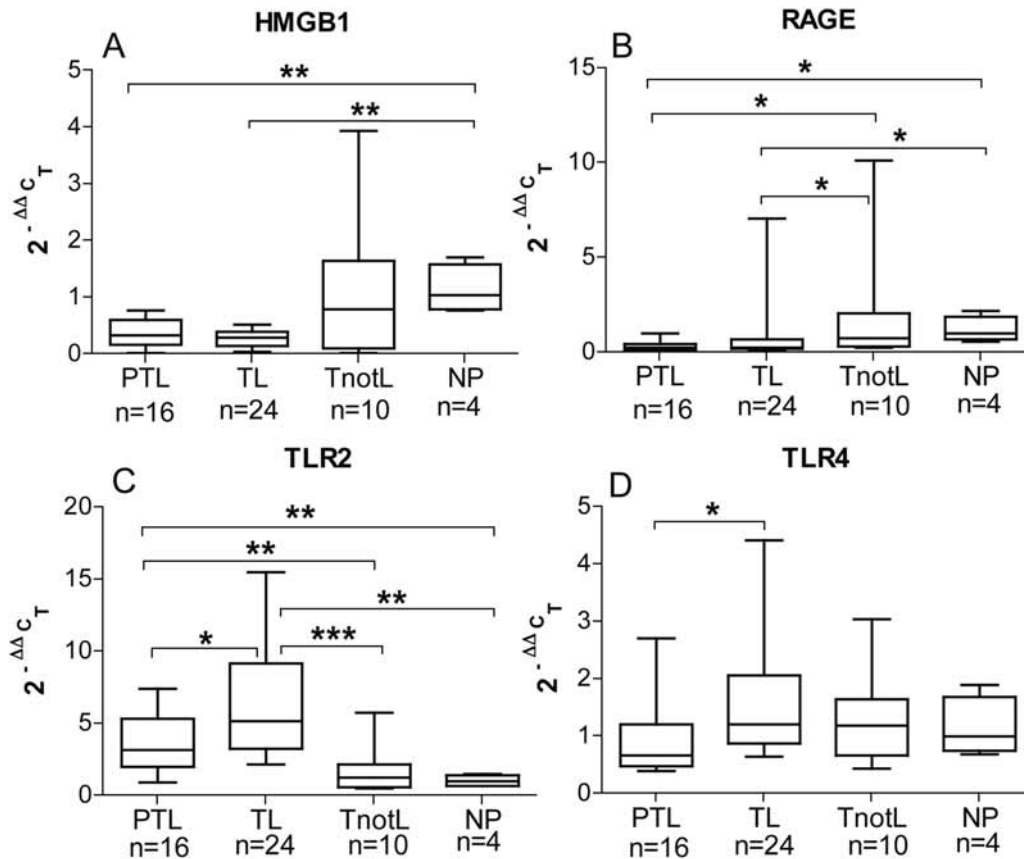


Fig. 4. — mRNA expression of HMGB1 (A), RAGE (B), TLR2 (C) and TLR4 (D) in cervical tissue. Preterm labour (PTL), term labour (TL), term not in labour (TnotL), non-pregnant (NP). The box represents 25-75% of all data with the line through the box representing the median value. The whiskers extend to the range (min/max). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Matrix metalloproteinases (MMPs)

The MMPs have broad and diverse substrate specificity: collagenases (MMP-1, -8 and -13) break down fibrillar and non-fibrillar collagens; stromelysins (MMP-3, -7 and -10) cleave proteoglycans, fibronectin, collagens IV, V and gelatins; gelatinases (MMP-2 and -9) target collagen IV, V, elastin, proteoglycan and fibronectin (Hulboy et al., 1997). Four tissue inhibitors of MMPs (TIMPs) have been described: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. The second group of MMP inhibitors are plasma α -macroglobulins (Hulboy et al., 1997). The levels of MMPs in cervix, lower uterine segment, amniotic fluid, placenta, foetal membranes and maternal plasma increase during labour (Osmers et al., 1995; Stygar et al., 2002; Sennstrom et al., 2003). Also, polymorphism in MMP-1 and MMP-9 genes is associated with PPRM (Ferrand et al., 2002).

In preterm cervical ripening MMP-1, MMP-3 and MMP-9 mRNA expression was up-regulated in labour and a tendency towards higher protein levels of MMP-8 and MMP-9 in labour has been found (Dubicke et al., 2008). A different secretion pattern at preterm and term was registered in vitro in cervical cell culture raised from cervical samples.

The secretion of IL-8 and MMP-1 was significantly higher ($p < 0.001$ and $p < 0.05$, respectively), but MMP-3 secretion significantly lower in preterm cervical fibroblasts compared to term fibroblasts ($p < 0.001$) (Dubicke et al., 2008) (Fig. 2).

Preterm cervical ripening

Although it is well established that intrauterine infection can lead to preterm labour, this does not appear to be the major cause of prematurity, since infection has been demonstrated in only 25-40% of preterm births (Slattery and Morrison, 2002; Goldenberg et al., 2008). Parturition itself is an inflammatory process. Inflammatory events can be observed in the myometrium, cervix, foetal membranes and peripheral blood (Tornblom et al., 2005a; Norman et al., 2007; Challis et al., 2009). Recent studies from our group indicate that cervical ripening at both term and preterm is an inflammatory process even if no infection is present (Tornblom et al., 2004; Tornblom et al., 2005a; Dubicke et al., 2008; Dubicke et al., 2010a; Dubicke et al., 2010b). The human cervix is dominated by ECM undergoing a pronounced remodelling both during preterm and term cervical ripening. Thus preterm cervical

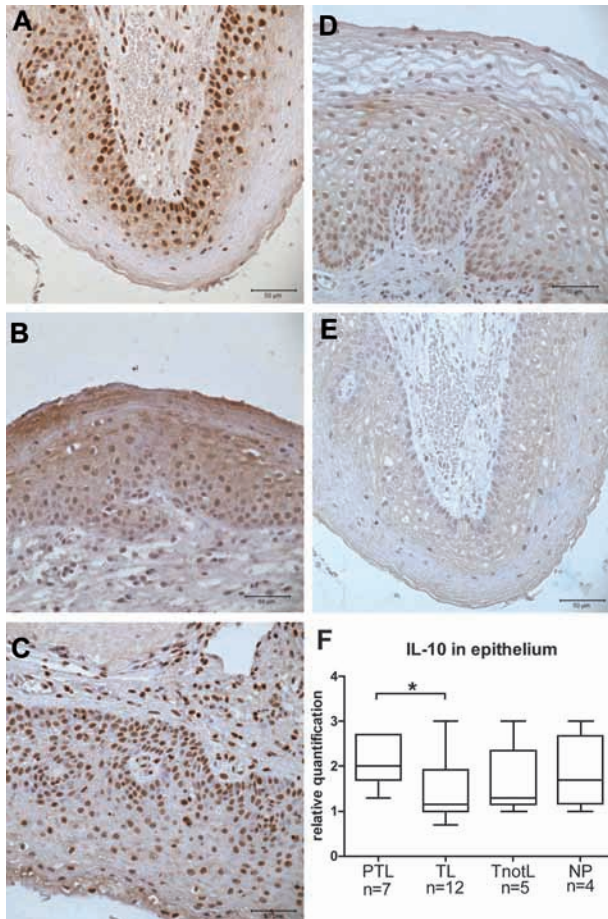


Fig. 5. — Immunohistochemical staining of IL-10 in cervical tissue. IL-10 in the cervical epithelium at preterm labour (A), term labour (B), term not in labour (C) and non-pregnant state (D). Negative control (E). Magnification $\times 400$. The mark is $50 \mu\text{m}$. Box and whisker plots represent the staining of IL-10 in the squamous epithelium (F). Preterm labour (PTL), term labour (TL), term not in labour (TnotL) and non-pregnant (NP). The box represents median value with 25%-75% of all data falling within the box. The whiskers extend to the range. The number of samples analyzed in each group is shown under the group name. A semiquantitative scale from 0 to +++ was applied. Statistically significant differences are indicated above the plot: * $p < 0.05$.

ripening seems to be a more relevant term than cervical insufficiency involved in preterm birth. The ultimate goal must be to develop new strategies to prevent preterm cervical ripening to reduce the number of spontaneous preterm births.

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