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Immunohistochemical expression of RECK protein in placental membranes of the preterm delivery with and without chorioamnionitis

Zdeslav Benzon[1], Ivana Kuzmiç Prusac[2], Sandra Zekic[2] and Marko Vulic[1]

[1]Department of Gynecology and Obstetrics and [2]Institute for Pathology, University Hospital Split, School of Medicine, University of Split, Croatia

Summary. Objective: To compare the immunohistochemical expression of RECK protein in placental membranes of late preterm delivery in women with and without histologically proven chorioamnionitis.

Study design: Fetal membranes were collected from women who had late preterm delivery with (n=8) and without (n=9) histologic chorioamnionitis. Immunohistochemistry for RECK protein was performed on formalin fixed and paraffin-embedded sections. The two groups were matched for age, body mass index and parity. SPSS Version 13.0 was used for statistical analysis.

Results: There was weaker immunohistochemical expression of RECK protein in placental membranes of women with histologic chorioamnionitis compared to control subjects (P=0.0498).

Conclusions: Chorioamnionitis has an impact on immunohistochemical expression of RECK protein in placental membranes in late preterm delivery.

Key words: Late preterm delivery, Placental membranes, Immunohistochemical expression, RECK protein

Offprint requests to: Marko Vulic, MD, Ph D, Department of Gynecology and Obstetrics, University Hospital Split, School of Medicine, University of Split,Spincica 1, 21 000 Split, Croatia. e-mail: marko.vulic1@st.t-com.hr

Introduction

Preterm deliveries are those that occur at less than 37 weeks of gestational age and account for 75% of perinatal mortality and more than a half of long-term morbidity. Preterm birth rate is 5-12 %, and depends on different factors (Goldenberg et al., 2008). Intrauterine infection is a frequent and important mechanism leading to preterm birth. Microbial endotoxins and proinflammatory cytokines stimulate the production of prostaglandins, other inflammatory mediators, and matrix-degrading enzymes. Prostaglandins stimulate uterine contractility, whereas degradation of the extracellular matrix in the fetal membranes leads to premature preterm rupture of membranes (pPROM) (Park et al., 2009; Menon et al., 2010). Chorioamnionitis leads to fetal and maternal inflammatory response which include proinflammatory cytokines like interleukin 1ß, 6 and 8 (IL-1ß, IL-6 and IL-8) and tumor necrosis factor alpha (TNFα) in fetal membranes and decidua (Ferrand et al., 2002; Keelan et al., 2003). An association between proinflammatory and anti inflammatory cytokines and activity of matrix metalloproteinase (MMP) and their inhibitors - tissue inhibitors of matrixmetalloproteinase (TIMP) has been studied in women with histologically proven chorioamnionitis and pPROM (Fortunato and Menon, 2001; Ferrand et al., 2002; Menon and Fortunato, 2007; Weidle et al., 2010; Menon et al., 2010). The results indicated that TNF-α and IL-1ß play a major role in promoting pPROM by inducing MMP activation and apoptosis with minimal effects induced by IL-6 (Fortunato and Menon, 2001; Fortunato et al.,

2002; Menon and Fortunato, 2007). Reversion-inducing cysteine rich protein with Kazal motifs (RECK) was initially discovered due to its ability to induce reversion in ras-activated fibroblasts. The product of the RECK gene can directly bind to a series of metalloendopeptidases, including MMP-2, MMP-9, and membrane type 1 (MT-1) MMP. The key action of RECK is to inhibit MMPs, especially MMP-2 and MMP-9 (Welm et al., 2002; Masui et al., 2003; Noda et al., 2003; Zhang et al., 2009; Miki et al., 2010).

All of the above suggests that RECK has an active role in extracellular matrix remodelling and has an inverse correlation with MMP-2 and MMP-9. So far there have been no investigations about RECK activity in fetal membranes. Based on current knowledge about MMP and TIMP activity in fetal membranes we expect an active RECK role. The aim of the study was to find out if there was a difference in immunohistochemical expression of RECK protein in placental membranes of women who had late preterm delivery (34-36$^{6/7}$ weeks of pregnancy) with and without histologic chorioamnionitis.

Materials and methods

Samples were obtained from seventeen placentas (eight without and nine with histologically diagnosed chorioamnionitis) delivered from 34 to 366/7 weeks of pregnancy. Chorioamnionitis has been histologically defined in the presence of acute inflammatory changes of a membrane roll and chorionic plate of the placenta (Redline, 2012). Control subjects were without chorioamnionitis. Gestational age was calculated by the first day of the last menstrual period or an early sonography. When a discrepancy occurred, ultrasonographic data were used. Investigated variables were immunohistochemical expression of RECK protein, maternal age, parity (primiparas and multiparas), body mass index (BMI; kg/m^2) and gestational age (days). Exclusion criteria were congenital anomalies, preeclampsia, Rh sensibilisation, polyhydramnios, gestational diabetes, cholestasis, pregnancies concieved after assisted reproductive technologies (ART) and multiple pregnancies. None of the patients had received any prostaglandin synthesis inhibitors or corticosteroids.

Diagnostic criteria for preeclampsia, gestational diabetes, polyhydramnios, obstetric cholestasis and IUGR were made according to definitions and recommendations from International societies.

Specimens of fetal membranes were obtained after cesarean section or vaginal delivery. Samples were taken from the rupture site to the place where the amnion starts to cover the placenta. All specimens were fixed in 4% buffered formalin, embedded in paraffin, stained with haemalaun-eosin and histologically analysed. For immunohistochemistry 4 μm sections were mounted onto superfrost plus glass (Termo Scientific, Germany). The immunohistochemistry was performed on Bench Mark Ultra Ventana autostainer (Roche Diagnostic, Germany). The primary antibody used in this study was monoclonal antibody against RECK protein diluted 1:50 (Abgent, San Diego, CA, USA). Ultra View Universal DAB detection kit (Ventana, Roche Diagnostic, Germany) was used as secondary antibody. Slides were counterstained with hematoxylin and covered with canada balsam. Each slide was analysed twice by an experienced pathologist who was blinded to the clinical diagnosis using a light microscope (Olympus BX41). The intensity of anti-RECK cytoplasmatic staining in fetal membranes was roughly graded into three: 0, undetectable; 1, weak; and 2, strong (Masui et al., 2003). Fig. 1a,b. If the results from the first and second testing did not match the slide was not taken into account. Statistical analysis was performed with Statistica SPSS version 13.0 (SPSS Chicago, IL) using the Student t-test, Fischer's exact and Chi square test. P values of <0.05 were considered statistically significant.

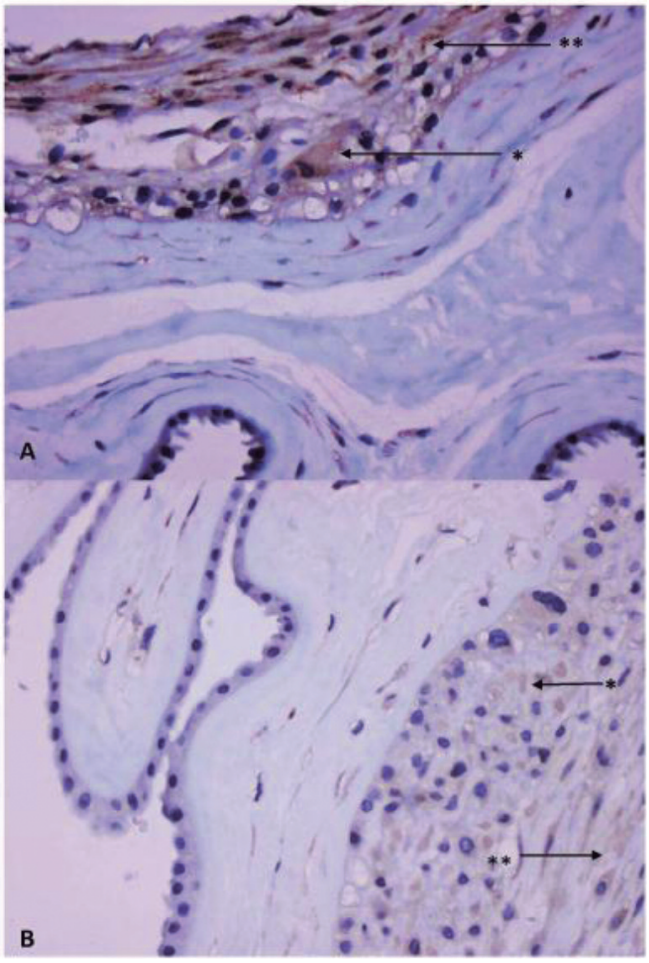

Fig. 1. A. Strong immunohistochemical expression of RECK protein in chorion (*) and decidua (**). **B.** Weak immunohistochemical expression of RECK protein in chorion (*) and decidua (**). x 400

Results

Placental membranes were collected from 17 women (eight with and nine without histologically diagnosed chorioamnionitis) who had late preterm delivery. All women with chorioamnionitis had maternal inflammatory response. Baseline characteristics of the women involved in the study are shown in table 1. Statistical analysis revealed no difference between investigated and control group in the terms of pPROM (χ^2=0.0413; P=0.8389), age (t=0.2392; P=0.8196), pregnancy duration (t=0.2106; P=0.8360) and parity (χ^2 =2.837; P=0.0921). However there was a difference in BMI (χ^2=6.4635; P=0.0394). (table 1).

Results of immunohistochemical expression of RECK protein in placental membranes with and without chorioamnionitis are shown in table 2. In the chorioamnionitis group, three of eight showed strong RECK expression (Fig. 1a) whereas five showed weak (Fig. 1b) or no expression. In the control group eight of nine women showed strong RECK expression, and one showed weak expression. The statistical analysis revealed a difference in RECK immunohistochemical expression between the chorioamnionitis and control group (P=0.0498). (Table 2).

Table 1. Baseline maternal characteristics of women who had late preterm delivery with and without chorioamnionitis.

Variables	Chorioamnionitis n (%) (N=8)	Controls n (%) (N=9)	P-value
pPROM	4	3	*P= 0.8389
No pPROM	4	6	χ^2=0.0413
Age (years ± SD)	31±44.12	29.33±5.7	**P=0.8196 t= 0.2392
Pregnancy duration (days ± SD)	250,25±4,1	251,67±5,2	**P=0.8360 t= 0.2106
Primiparas	3 (37,5)	7 (77,78)	*P=0.0921
Multiparas	5 (62,5)	2 (22,22)	χ^2=2.837
BMI***(kg/m^2)			
18.5-24.9	5 (62,5)	3 (33,33)	*P=0.0394
25-29.9	0	5 (55,56)	χ^2=6.4635
≥30	3 (37,5)	1 (11,11)	

pPROM: premature preterm rupture of membranes; *: Chi square test; **: Student t test; ***: BMI - Body mass index.

Table 2. RECK expression in chorion and decidua of women with late preterm delivery with and without chorioamnionitis.

	Chorioamnionitis	Controls	*P value
Weak or no expression	5	1	P=0.0498
Strong expression	3	8	

*: Fisher's exact test.

Discussion

This study has shown for the first time that there is an immunohistochemical expression of RECK protein in placental membranes of women who delivered from 34 to $36^{6/7}$ weeks of pregnancy. The results also showed that chorioamnionitis had an impact on RECK immunohistochemical expression in chorion and decidua of placental membranes in late preterm delivery. This observation is in concordance with previously published data about MMP especially, MMP-2 and MMP-9, expression and activity in fetal membranes of women with chorioamnionitis (Xu et al., 2002; Goldman et al., 2003; Weiss et al., 2007; Menon and Fortunato, 2007).

There are several disadvantages of our study. The main disadvantages of our study are small sample size, immunohistochemistry as semiquantitative method for evaluating protein presence, and possible subjectivity of investigators. Due to the strict exclusion criteria the first disadvantage was very hard to avoid. We tried to avoid subjectivity of the investigator by blindness to clinical data. Analysis of each slide stained with RECK was performed twice by the experienced pathologist. If there was a disagreement between two examinations about the results of the immunohistochemical analysis, specimens were excluded from the study. Finally, analyses were performed on placental membranes after establishing uterine contractions and there was no data about association between contractions and RECK expression.

Based on the known risk factors and the four postulated mechanistic pathways of preterm birth -PTB (infection/inflammation, stress, decidual hemorrhage, and uterus overdistention) chorioamnionitis plays a major role (Lockwood and Kuczynski, 2001). Intrauterine infection is accompanied by a host-inflammatory response, which involves an accumulation of inflammatory cells in the placental membranes and expression of cytokines in the fetoplacental tissues (Keelan et al., 2003; Menon et al., 2010). Cytokines are known to modulate a variety of parturition-related processes, including cervical ripening via promotion of synthesis of nitric oxide, prostaglandins, and matrix metalloproteinases; myometrial activity via regulation of cyclooxygenase-2 dependent prostaglandin production; and finally, preterm birth, which can be induced by exogenous IL-1ß or TNF-α (Bollapragada et al., 2009).

The histology of fetal membranes is characterised by the abundant proportion of the connective tissue in both the amnion and the chorion. Although the chorion is thicker than the amnion, the tensile strength of the membranes is attributed mainly to the compact layer of the amnion, its name derived from the tight arrangement of abundant collagen fibres and the absence of amnion cells (Bryant-Greenwood, 1998; Menon and Fortunato, 2007). The reticular and spongy layers of the chorion also contribute to mechanical support. The amniochorionic extracellular matrix is composed of several different genetic types of collagen arranged in a complex framework, maximising its mechanical

resistance. Major components are types I, III, IV, V and VI collagens and abundant proteoglycans, which are embedded in the fibrous proteins. It is widely accepted that the principal tissue support is generated by fibres composed of types I and III collagens, which themselves are stabilised by a network of collagen types: IV, V and VI. These structures lose their architecture and mechanical strength around labour synchronously with increased myometrium activity and cervical ripening, allowing delivery to occur (Vadillo-Ortega and Estrada-Gutierrez, 2005).

Most of the ECM and basement membrane components can be degraded by matrix metalloproteinases (MMPs), a group of structurally related, zinc-dependent enzymes. Even though the specific role of all MMPs in the degradation of the amniochorionic extracellular matrix has not been described, it is now evident that a number of substances composed of at least gelatinases, collagenases and stromelysin are expressed during amniochorionic rupture. Major enzymes associated with this process are gelatinases A and B (MMP-2 and MMP-9, respectively) and collagenases 2 and 3, also known as MMP-8 and MMP-13. Other MMPs such as stromelysin-1 (MMP-3) are expressed during labour (Vadillo-Ortega and Estrada-Gutierrez, 2005).

Gelatinases have been identified in human fetal membranes and amniotic fluid. An increase in MMP-9 levels in fetal membranes and amniotic fluid has been associated with term labour, indicating a role for MMP-9 in human parturition (Xu et al., 2002). During the infection there is a documented imbalance between MMP and TIMP, which might indicate matrix metalloproteinase activity causing destruction of extracellular matrix components, rather than remodelling (Goldman et al., 2003; Vadillo-Ortega and Estrada-Gutierrez, 2005; Weiss et al., 2007). However, there is a little information concerning changes in MMP level with spontaneous preterm labour, and the cell sites of MMP-2 and MMP-9 expression are not clearly known (Vadillo-Ortega and Estrada-Gutierrez, 2005).

In media conditioned by chorion, the initiation of uterine contractions did not change MMP activity or TIMP-1 levels. However, an increase in MMP-9 activity and a decrease in TIMP-1 protein levels were observed following the establishment of uterine contractions in media conditioned by amnion (Goldman et al., 2003; Vadillo-Ortega, and Estrada-Gutierrez, 2005).

The RECK gene has been identified as a negative transcriptional target of molecules, including multiple retroviral oncogenes and EBV latent membrane protein 1 (Miki et al., 2010). It is widely expressed in various human tissues and non-neoplastic cell lines (Zhang et al., 2009; Miki et al., 2010).

RECK is an important regulator of extracellular matrix remodelling and the down-regulation of RECK by oncogenic signalling leads to the excessive activation of MMPs (Noda et al., 2003). The RECK protein regulates at least three members of the matrix metalloproteinase (MMP) family, MMP-2, MMP-9, and MT1-MMP, in vitro or in cell cultures. It attenuates their proteolytic activity competitively in most cases (Miki et al., 2010). Data suggest that RECK can inhibit MMPs through several mechanisms, including direct inhibition of protease activity, regulation of their release from the cell and possibly through sequestration of MMPs at the cell surface (Welm et al., 2002; Noda et al., 2003; Miki et al., 2010). Because of its structural feature, RECK controls metalloendopeptidase activities in limited distance from the membrane surface (Noda et al., 2003; Miki et al., 2010) Finally, our current knowledge about interactions between RECK and specific MMP and TIMP is insufficient. For instance, in pancreatic cancer tissues, RECK expression showed a significant inverse correlation with MMP-2 activation but not with MMP-9 activation (Masui et al., 2003). MMP-9 (gelatinase B) is an inducible gene in human fetal membranes, the expression of which increases during infection, PROM, and active term labor (Vadillo-Ortega and Estrada-Gutierrez, 2005). We have not found similar data about RECK gene inducibility.

Recent investigations suggest that a single nucleotid polimorphism (SNP) involved in controlling fetal inflammation (interleukin-6 receptor 1 –IL6R) and DNA variants in maternal genes encoding for proteins involved in extracellular matrix metabolism approximately doubled the risk of PTB. The main observations were that in mothers a SNP in TIMP2 was significantly associated with the phenotype and in fetuses a SNP in IL6R increased the risk for preterm labour/delivery. Also, the haplotypes for alpha 3 type IV collagen isoform 1 (COL4A3) in mother and Insulin-like growth factor 2 (IGF2) and Interleukin 2 precursor (IL2) in fetus were associated with preterm labour/delivery with intact membranes (Romero et al., 2010).

There are data that show that there is a diversity in relative expression of cytokine and chemokine genes in amnion and choriodecidual membranes by cDNA arrays. In the group with preterm labour and chorioamnionitis relative expression of IL6 and TNF· was higher than in the group with preterm labour without infection (Keelan et al., 2003). It has been shown that there is a diversity of cytokine response to the bacteria associated with preterm birth in fetal membranes. Fetal membrane cytokine signatures of four different bacteria associated with PTB are distinct, suggesting that infection as a potential cause of PTB is not homogeneous in its presentation (Menon et al., 2009). It seems that there are two divergent molecular pathways for premature rupture of membranes and preterm labour (Fortunato and Menon, 2001). Differences in apoptosis and MMP activity in fetal membranes caused by different proinflamatory cytokines are involved in pPROM and spontaneus preterm delivery. The findings suggest that one or more cytokines may act as a switch between the two pathways leading to pPROM in some women and preterm labour without ROM in others (Fortunato and Menon, 2001; Menon et al., 2010).

In conclusion, the impact of infection on collagen metabolism, MMP, TIMP and RECK activity in fetal membranes in women who had late preterm birth is still controversial. This study introduces RECK as a new factor which in theory could have an impact by weakening the amniotic and choriotic extracellular matrix (ECM) by degradation and a reducing collagen content, which is one of the key events predisposing the membrane to rupture. Future investigations should contribute to better understanding of pPROM etiology and spontaneus preterm birth without rupture of membranes in late preterm delivery.

Increasing sample size, Western Blot, zymography and PCR for RECK are the mandatory aims of the future studies that could bring a more precise definition of its possible physiological activity in fetal membranes. Accumulating evidence suggests that labour is an inflammatory process, so it is also important to find out if RECK is an inducible gene in human fetal membranes.

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