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The MMP-9/TIMP-1 imbalance and the reduced level of TGF-β in the cervical area of amniotic membrane is a possible risk factor of PROM and premature labor — proof-of-concept study

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ABSTRACT

Objectives: To assess the level MMP-9, TIMP-1 and TGF-β in placental and cervical region of amniotic membranes derived from at-term, pre-term and PROM deliveries.

Material and methods: 14 amniotic membranes have been assessed; the quantitative analysis of MMP-9, TGF-β and TIMP-1 was assayed using respective Quantikine Immunoassay Kit.

Results: The MMP-9 level in PROM samples was similar to the level of MMP-9 in at-term membranes and comparable between the cervical and placental region of these membranes. The concentration of TGF-β and TIMP-1 was decreased in the cervical area of AM derived from deliveries complicated with PROM.

Conclusion: The MMP9/TIMP-1 imbalance, as well as the reduced level of TGF-β may be possible risk factors of pre-term labor and PROM.

Key words: amniotic membrane, PROM, pre-mature labor, TGF-β

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INTRODUCTION

The premature rupture of fetal membranes (PROM) is one of the mechanisms leading to pre-term delivery that is a well-known risk factor for the increased morbidity and mortality of the neonates [1]. It is postulated that the amnion rupture is not only the consequence of a simple stretching by mechanical forces caused by contracting uterus. It is considered rather as a biologically programmed process of the histological remodeling leading to membrane weakening [2]. Studies conducted by different authors led to the conclusion that the amniotic membrane is not a homogenous tissue and that at least two different areas of the amnion: placental and cervical (apical), can be distinguished [3, 4]. The placental region, the area distant from the uterine cervix, that covers the placenta, is the thickest part of the amnion, particularly resistant to stretch forces. The cervical area, often described as a "zone of extreme morphology" is the region overlying the cervix, where amnion ruptures prior to delivery. Several studies have confirmed that this region contains less epithelial cells and is significantly thinner and weaker than the rest of AM [5, 6]. Physiological mechanisms involved in AM weakening in a course of gestation remain unknown [7–9]. Nevertheless, it has been postulated that biologically active factors responsible for extracellular matrix (ECM) turnover, especially matrix metalloproteinases (MMPs) and their inhibitors play a crucial role in this process. MMPs belong to a large family of zinc endoproteinases. They are able to cleave main ECM components, including collagens,

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elastin, fibronectin, gelatin and aggrecan. MMP-9, or gelatinase B, is mainly produced by macrophages and neutrophils. Subsequently to proteolytic activation by a variety of factors, including plasminogen activators and other MMPs (MMP-2, -3, -12), MMP-9 displays elastolytic, collagenolytic and gelatinolytic activity [10]. Whereas some matrix metalloproteinases, as MMP-2 are produced constitutively in fetal membranes, the production and release of MMP-9 increase under specific conditions, such as inflammation and the onset of labor [11]. The role of inflammation in the release of the proteolytic enzymes and ECM degradation should be taken into consideration, nevertheless some cases of PROM are not related to any intrauterine infection [12]. Therefore, it is plausible that other mechanisms may be involved in the premature membrane weakening. The family of tissue inhibitors of metalloproteinases (TIMPs) consists of four protease inhibitors, secreted by different types of cells, like macrophages, vascular smooth muscle cells and platelets. They suppress the MMPs activity by binding to their catalytic domain and blocking enzymatic properties [13]. Another cytokine that plays an important role in the regulation of MMPs activity and extracellular matrix remodeling is transforming growth factor β (TGF-β) [14]. It is plausible that changes in the level of these cytokines may lead to the increase of MMP proteolytic activity and lead to the AM rupture. In our previous study, concerning AM obtained after physiological deliveries at term, we have shown that the content of TIMP-1, as well as TGF-β is different in placental and cervical region of the AM [15]. There is a limited number of studies concerning TIMP-1 and TGF-β content in AM derived from pre-term deliveries or pregnancies complicated with PROM and the results remain inconsistent [16, 17]. Moreover, none of these studies take into consideration the differences between placental and cervical area of the membrane.

OBJECTIVES

The aim of our present study was to investigate the level MMP-9, TIMP-1 and TGF-β in placental and cervical region of amniotic membrane samples derived from at-term and pre-term deliveries as well as deliveries complicated with PROM.

MATERIAL AND METHODS

Amniotic membrane samples collection and preparation

14 membranes have been collected in the Department of Obstetrics and Gynecology of Warsaw Medical University Hospital. 6 AM originated from at-term physiological deliveries, 5 AM from pre-term deliveries and 3 AM from deliveries complicated with PROM. A pre-term delivery was defined as a completed labor that started before 37 weeks of pregnancy. PROM was defined as a rupture of membranes in a patient who was beyond 37 weeks' gestation, prior to the onset of labor (uterine contractions leading to changes in the cervix). Additionally, clinical data about past obstetric history, comorbidities, medications and infections during present pregnancy have been collected for each patient. Written informed consent was obtained from all donors of amnion samples. The concept of the study was reviewed and approved by the local bioethics committee. The study procedures conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki.

From each amniotic membrane sample a cervical and placental portion has been separated, according to the method described previously. Small fragments of approximately 1 cm^2 have been used to prepare amniotic membrane extracts. Samples were immersed in phosphate-buffered saline (PBS) with 1% Triton X-100 and a mixture of broad range protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany) and mechanically dispersed using glass homogenizer. The obtained suspensions have been centrifuged, and supernatants were transferred into fresh tubes, and stored until being used for further analysis.

ELISA

The quantitative analysis of MMP-9, TGF-β and TIMP-1 in amniotic membrane extracts was assayed using respective commercially available Quantikine Immunoassay (R & D Systems Inc. Minneapolis, USA). The level of absorbance for each tested sample was measured using the Microplate Reader 550 (BIO-RAD, Hercules, CA). The concentrations of respective cytokines were calculated based on corresponding standard calibration curve and expressed in ng (TIMP-1, MMP-9) or pg (TGF-β) per mL. The assay sensitivity for each factor was respectively: 0.08 ng/mL for TIMP-1, 0.156 ng/mL for MMP-9 and 15.4 pg/mL for TGF-β.

RESULTS

The clinical characteristic of all the patients included in the study is presented in Table 1.

TGF-β concentration in at-term, pre-term delivery and PROM-derived membranes

TGF-β was present in all tested amnion samples (Fig. 1A).

The mean concentration of TGF-β in extracts prepared from pre-term labor derived membranes was similar between cervical and placental region: 156 ± 184.1 pg/mL (median 57.2 pg/mL, min. 15.5 – max. 453.9) *vs.* 157.5 ± 133.9 pg/mL (median 99.8 pg/mL, min. 70 – max. 391.7) respectively. Mean concentration of TGF-β was 115.5 ± 108.7 pg/mL (median 64 pg/mL, min. 18.9 – max. 266.6) in placental region, *vs.* 119.8 ± 96.5 pg/mL (median 100.2 pg/mL, min. 31.7 – max. 289.6) in cervical

Table 1. Clinical characteristics of all patients included in the study

	Pre-term labor	PROM	At-term labor
Median maternal age			
	29 years (26–38)	32 years (29–32)	33.5 years (26–37)
Median gestational age			
	36 Hbd (34–36)	38 Hbd (37–39)	39.5 Hbd (37–40)
Pap test result			
Normal	1	1	2
ASCUS*	2	2	3
Not known	2	0	1
GBS test result**			
Positive	1	0	0
Negative	3	3	6
Not known	1	0	0
Other confirmed local infections			
Mycotic	1	0	0
None	4	3	6
Reason for pre-term labor			
Signs of fetal hypoxia	1	–	–
Pre-eclampsia	1		
Not known	3		

*Atypical squamous cells of undetermined significance
**Group B Streptococcus

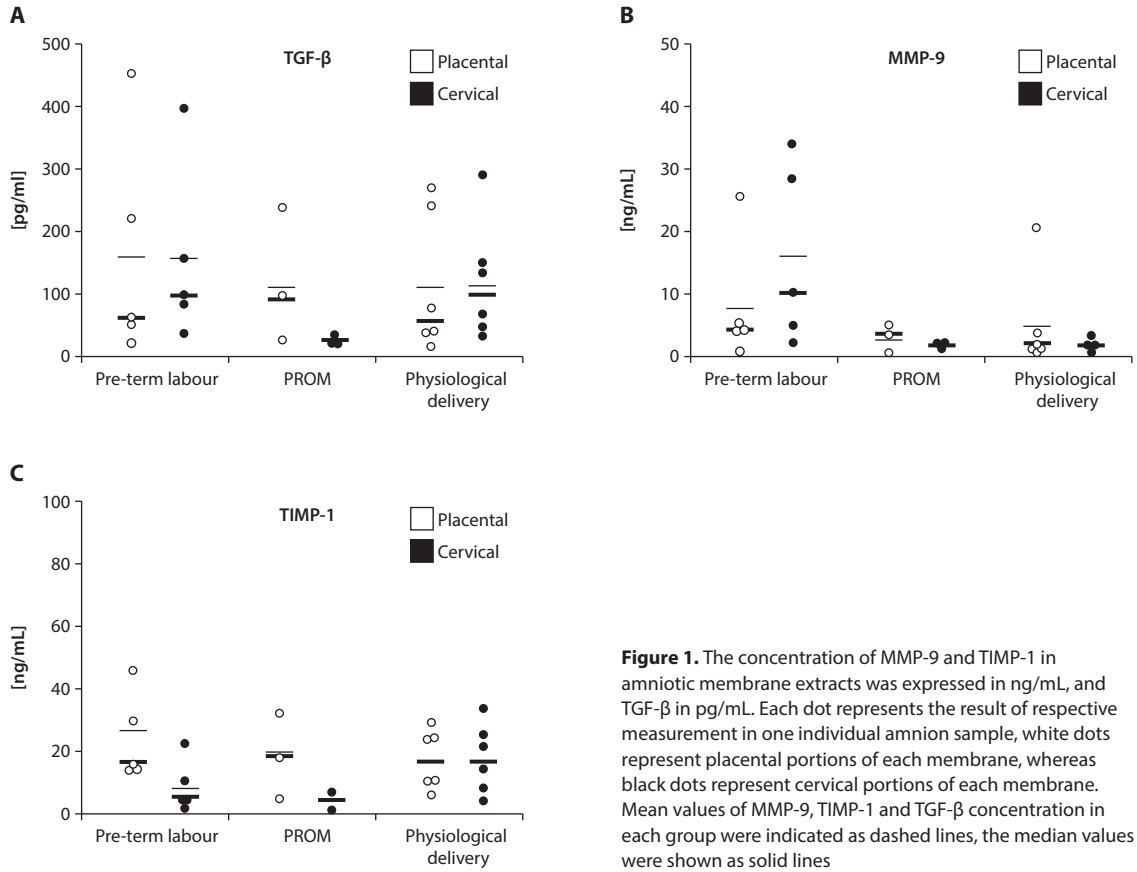

Figure 1. The concentration of MMP-9 and TIMP-1 in amniotic membrane extracts was expressed in ng/mL, and TGF-β in pg/mL. Each dot represents the result of respective measurement in one individual amnion sample, white dots represent placental portions of each membrane, whereas black dots represent cervical portions of each membrane. Mean values of MMP-9, TIMP-1 and TGF-β concentration in each group were indicated as dashed lines, the median values were shown as solid lines

region. The mean concentration of TGF-β in placental region of PROM samples was 116.8 ± 106.5 pg/mL (median 94.6 pg/mL, min. 23.1 – max. 232.6). The level of TGF-β in cervical region of these membranes was 20.3 ± 2.2 pg/mL (median 20.6 pg/mL, min. 18.0 – max. 22.3).

MMP-9 concentration in at-term, pre-term delivery and PROM-derived membranes

The quantitative analysis using ELISA tests has confirmed the presence of MMP-9 in all tested AM samples (Fig. 1B).

The mean concentration of MMP-9 in extracts prepared from placental region of pre-term membranes was 8.7 ± 10.0 ng/mL (median 4.9 ng/mL, min. 1.4 – max. 26.3) and in cervical region 16.1 ± 14.3 ng/mL (median 10.5 ng/mL, min. 2.7 – max. 33.7). In PROM group mean concentrations of MMP-9 in placental and cervical region were 3.3 ± 2.6 ng/mL (median 4.4 ng/mL, min. 0.3 – max. 5.3) and 1.6 ± 0.1 ng/mL (median 1.6 ng/mL, min. 1.5 – max. 1.7) respectively. The mean concentration of MMP-9 in at-term labor derived membrane extracts from placental region was 5.7 ± 8.8 ng/mL (median 1.8 ng/mL, min. 0.9 – max. 23.4) whereas in cervical region 2.5 ± 1.2 ng/mL (median 2.5 ng/mL, min. 1.1 – max. 3.9).

TIMP-1 concentration in at-term, pre-term delivery and PROM-derived membranes

The immunoenzymatic assessment has revealed the presence of TIMP-1 in all tested samples (Fig. 1C). The mean concentration of TIMP-1 in pre-term labor derived membrane extracts from placental region was 25.2 ± 13.0 ng/mL (median 17.4 ng/mL, min. 15.7 – max. 45.2) whereas in cervical region 9.8 ± 7.7 ng/mL (median 7.6 ng/mL, min. 2.8 – max. 22.7). In at-term derived membranes mean concentration of TIMP-1 was 18.3 ± 9.4 ng/mL (median 18.4 ng/mL, min. 6.7 – max. 30) in placental region and 18.2 ± 11.3 ng/mL (median 18.4 ng/mL, min. 4.3 – max. 33.8) in cervical region. In PROM samples mean concentration of TIMP-1 in placental and cervical region was 20.3 ± 16.4 ng/mL (median 19.2 ng/mL, min. 4.5 – max. 37.3) *vs.* 4.5 ± 3.4 ng/mL (median 5.9 ng/mL, min. 0.7 – max. 7.0) respectively.

MMP-9/TIMP-1 ratio

The MMP-9/TIMP-1 ratio was calculated for each study group (Fig. 2). The mean MMP-9/TIMP-1 ratio in extracts prepared from placental region of pre-term membranes was 0.44 (median 0.14) and in cervical region 1.81 (median 1.62). In PROM group mean MMP-9/TIMP-1 ratios in placental and cervical region were 0.15 (median 0.12) and 0.98 (median 0.29) respectively. The mean MMP-9/TIMP-1 ratio in at-term labor derived membrane extracts from placental region was 0.32 (median 0.19) whereas in cervical region 0.21 (median 0.16).

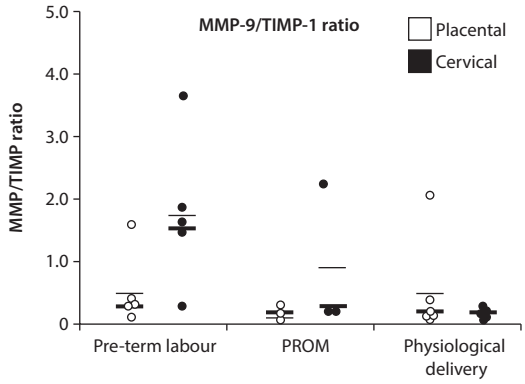

Figure 2. The correlation between MMP-9 and TIMP-1 in amniotic membrane extracts was expressed as a MMP-9/TIMP-1 ratio. Each dot represents the result of respective measurement in one individual amnion sample, white dots represent placental portions of each membrane, whereas black dots represent cervical portions of each membrane. Mean values of MMP-9/TIMP-1 ratio in each group were indicated as dashed lines, the median values were shown as solid lines

DISCUSSION

The results of our proof-of-concept study have shown that the cervical area of pre-term derived AM is characterized with the reduced level of TIMP-1 and the increased level of MMP-9 in comparison to corresponding region of membranes collected after physiological at-term deliveries. At the same time cervical region of PROM derived amnions is characterized by lower amounts of TIMP-1 and TGF-β than the cervical area of AM obtained from deliveries not complicated with PROM. Despite the limited number of samples included in the study this observation may help to explain the pathophysiological mechanisms of pre-mature membrane rupture. Therefore, it might be interesting to investigate it on a larger group of patients.

Since the cervical area of AM is the region where the amnion ruptures prior to delivery and that displays different histological properties than the rest of the membrane, we hypothesized that any changes in MMP-9, TIMP-1 or TGF-β levels during course of gestation would predominantly manifest in the cervical region of the AM. The early studies on changes in AM tensile strength and collagen concentration failed, until the authors took into consideration the differences between placental and cervical portion of the membrane [12, 18]. In our study the level of MMP-9 in PROM samples was similar to the level of MMP-9 in at-term membranes, whereas in pre-term group it was higher in cervical area in comparison to the placental region. The overall content of MMP-9 in pre-term membranes was also higher when compared to amnions collected after at-term physiological deliveries. At the same time the level of TIMP-1 was reduced in the cervical area of PROM as well as pre-term derived membranes, in comparison to the placental area. It was also lower than in corresponding area of membranes

obtained after physiological deliveries at-term (data from our previous study). Several authors have investigated the level of TIMP-1 in the amniotic fluid in pre-term deliveries and PROM with inconsistent results [19]. Locksmith et al. have observed the decreased level of TIMP-1 in PROM samples, which was consistent with the previous study of Vadillo-Ortega et al. [20, 21]. Another group reported elevated TIMP-1 levels in amniotic fluid samples derived from deliveries complicated with PROM, nevertheless this study did not include MMP-9 assessment or MMP-9/TIMP-1 ratio [22]. The results of studies mentioned above are of a limited practical value since the authors did not take into account the heterogeneity of the AM. The results of our study show that significant differences in the amount of TIMP-1 between PROM and non-PROM membranes appear mainly in the cervical region of AM. Therefore, the mean concentration of this factor assessed in a membrane as a whole, may not reflect the real variances between samples derived from pre-term and at-term ruptured membranes. Moreover, the concentration of TIMP-1 in the amniotic fluid depends not only on its presence in the AM but also on placental and fetal secretion of this factor.

It has been proven that in pathological processes a physiological balance between MMPs and TIMPs is disturbed, that as a consequence leads to the impaired ECM turnover [12]. Therefore, the MMP-9/TIMP-1 ratio might be a better indicator of ECM remodeling than an absolute level of MMP-9 or TIMP-1 in the assessed tissue sample. In our study in pre-term membranes study group the mean MMP-9/ /TIMP-1 ratio was significantly higher in cervical than in placental region of AM, which may indicate the predominance of ECM degradation processes over synthesis and may be a hallmark of membrane weakening. A similar, although not that strong effect was observed in PROM membranes, which may be a consequence of a limited number of samples assessed in this study group.

The results of our study suggest, that the reduced level of TGF-β in the cervical area of AM may be one of the possible risk factors of premature membrane weakening. In our study we have observed the overall lowest amounts of TGF-β in the cervical area of membranes collected from PROM-complicated deliveries. As mentioned before, TGF-β is a multifunctional cytokine that is known to regulate cells proliferation and differentiation, inflammation, angiogenesis, as well as ECM remodeling in a variety of tissues and organs, also during embryonic development [14]. The potential role of TGF-β in human parturition has not been fully explained [23]. Nevertheless, since TGF-β regulates the inflammatory response and may have an influence on MMPs activity as well as TIMPs expression, it is plausible that its reduced level plays an important role in the creation of a weak zone in the cervical area of AM prior to delivery. The exact physiological mechanisms associated with TGF-β signaling that are responsible for AM weakening and subsequent rupture need to be elucidated.

CONCLUSIONS

The results of our proof-of-concept study suggest that the MMP-9/TIMP-1 imbalance, as well as the reduced level of TGF- β in the cervical area of AM, may be one of possible risk factors of pre-mature AM rupture. The observed imbalance of biologically active factors concentration in the cervical area of pre-term and PROM membranes may help to reveal the physiological mechanisms that are involved in AM weakening process. Nevertheless, the study was conducted on a limited number of samples, therefore, these interesting preliminary observation require further investigation, on a larger group of patients.

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