Changes in Matrix Metalloproteinase (MMP)-2 and MMP-9 in the Fetal Amnion and Chorion During Gestation and at Term and Preterm Labor

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Increased matrix metalloproteinase (MMP)-9 proteolytic activity is associated with term birth, preterm birth and premature rupture of membranes. However, most studies show no changes with MMP-2, which binds tightly to cell and matrix proteins. We hypothesized better protein extraction would reveal new MMP patterns. Human amnion and chorion were collected from 25 patients at preterm or term, extracted with 2% SDS (a high concentration), and the MMP protein levels and pro-enzyme activities were determined by Western immunoblotting and zymography. MMP-2 protein and MMP-2 and -9 pro-enzyme activities in the amnion increased significantly ($p < 0.05$) with labor at term, and were higher than at preterm labor ($p < 0.05$), when extracted with high SDS concentration. There were no changes in chorion MMPs under any condition. These associations suggest MMP-2 may be another regulator of membrane rupture and other labor-associated mechanisms at term parturition, and its role(s) should be examined further.

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INTRODUCTION

Extensive extracellular matrix remodeling is critical in many birth-related physiological processes such as cervical ripening, fetal membrane rupture, and placental detachment [1–5]. Matrix metalloproteinases (MMPs), a group of zinc-dependent enzymes, play important roles in such remodeling. Specifically, MMP-2 and MMP-9, known as gelatinase A and B, respectively, are capable of degrading collagen type IV, elastin, and fibronectin and have been identified in the fetal membranes, decidua, and amniotic fluid. Several earlier reports showed that an increase in MMP-9 protein and activity in the fetal membranes, placenta, and amniotic fluid is associated with fetal membrane rupture, term and preterm parturition, and placental detachment from maternal tissue, thus suggesting a role for MMP-9 in these labor-associated events. Similar changes in MMP-2 protein and activity are usually not reported [5–9], leading to the conclusion that MMP-2 is constitutively expressed during gestation, whereas MMP-9 expression is induced by active labor, infection, or premature rupture of the membranes [6]. Only one observation has indicated an increase in MMP-2 [8].

The regulation of MMP activity is, however, complex. Following their production, MMPs may be tightly bound to the cell surface or the extracellular matrix, with concurrent low proteolytic activity. After activation, these MMPs detach from the cell surface or the extracellular matrix to affect their proteolytic activity [10]. It is possible that these MMPs are bound with various degrees of avidity, suggesting the intriguing possibility that MMPs may exist in at least two states – free or loosely bound and tightly bound MMPs – and that routine protein extraction procedures using 0.1% sodium dodecyl sulfate (SDS) may be isolating only the free or loosely bound MMPs. Such a possibility has been suggested by Yu and Woessner [10] for MMP-7 in the uterus.

The aim of this study, therefore, was to determine whether extracting with a higher concentration of the anionic detergent, sodium dodecyl sulfate (SDS), would reveal new expression...
and/or activity patterns of gelatinases in the fetal membranes during gestation and at term and preterm birth. We hypothesized that more efficient protein extraction would demonstrate changes with MMP-2 with parturition.

MATERIAL AND METHODS

Tissue collection

Fetal membranes were obtained from a total of 25 patients at preterm (26–34 weeks of gestation) patients in labor and not in labor and from term (37–41 weeks of gestation) patients in labor and not in labor using procedures approved by the institutional ethics committee, which includes written consent obtained from the patients before delivery of their babies. All non-labored tissues were collected at cesarean section whereas all other tissues were obtained following spontaneous vaginal delivery. Tissues from patients with documented inflammation or infection determined by histological examination of neutrophil invasion by a pathologist were excluded.

The amnion and chorion were isolated immediately after delivery by one of the investigators. Briefly, the membranes were cut from the basal plate and rinsed in 250 mL of 4 °C saline solution. The samples were then placed on a dissecting tray, and the amnion was separated from the chorion by scraping. We are aware that this procedure does not remove all of it from the chorion. Each tissue type was rinsed in 4 °C saline solution and divided into approximately 1 g portions. All tissues were snap frozen in liquid nitrogen and stored at −80 °C until processed. Tissues for the concentration–response test of SDS extraction were not separated and combined amnio-chorion were used.

Protein extraction

To each tube containing 800 mg of tissue, 6 mL of extraction buffer (50 mM Tris, pH 7.4, 10 mM CaCl₂, 0.25% Triton X-100, 0.03% sodium azide, 1 mM Na-orthovanadate, and 50 μL Protease Inhibitor Cocktail, Sigma Chemical Co., St. Louis) was added. Contents were homogenized with short bursts at 4 °C. To test the extraction potential of SDS in initial experiments, increasing concentrations of SDS (0, 0.1, 0.2, 0.5, 1.0 or 2.0%) were added to samples, or following the test, the proficient SDS (0.1% SDS) was performed with 0.05% (w/v) Coomassie brilliant blue G-250 in 25% (v/v) methanol plus 10% acetic acid for 1 h and destained in 4% (v/v) methanol plus 8% (v/v) acetic acid for 2 h. After identification, quantification of the bands corresponding to 72-kD and 92-kD gelatinases (pro-forms) was performed with a densitometer. We recognize that physical agents such as the 7.5% SDS in the gel unfold the pro-form structure to expose the zinc moiety leading to activation of the metalloproteinase which then is able to digest the gel [11]. Therefore, this is not a true measure of endogenous gelatinase enzymatic activity, but rather an estimation of pro-enzyme mass based upon proteolytic activity. To avoid confusion throughout the manuscript with mass obtained from Western immunobLOTS, we will refer to data obtained from zymographs as “pro-enzyme activity”.

Western blot analysis

The abundance of MMP-2 and MMP-9 protein in the tissues after extraction with 0.1% or 2.0% SDS (or with increasing concentrations of SDS in the initial test (Figure 1)) was assessed by Western blot analysis. Samples (40 μg protein per lane) were size fractionated on a 10% (v/v) SDS–polyacrylamide gel under non-reducing conditions and transferred onto a nitrocellulose membrane (pore size 0.45 μm; Biorad) in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol, and 0.1% SDS) at 100 V for 1 h at 4 °C. After the blots were stained to detect the presence of protein, they were washed with 0.1% TBS–T (TBS: 50 mM Tris–HCl [pH 7.5], 200 mM NaCl), and were blocked with 7% skim milk powder in 0.1% TBS–T overnight at 4 °C.

The blots were then incubated with antibodies to MMP-2, MMP-9 (Sigma Co, St. Louis MO, USA), or β-actin (Neomarkers, Fremont, Canada) at a dilution of 1:1000 in blocking solution for 2 h at room temperature. The blots were rinsed five times for 15 min each time with 0.1% TBS–Tween and incubated with secondary antiserum conjugated with horseradish peroxidase for 1 h (1:2000 dilution in blocking solution). After appropriate washing, protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, UK) and exposed to X-ray film (Fuji Medical Film, Fuji Co, Tokyo, Japan). After binding for MMP-2, the blots were washed and re-probed for MMP-9. Clear bands at the appropriate molecular weights were evident (data not shown), indicating the antibodies were specific. Relative intensities of MMP-2, MMP-9, and β-actin were derived from the Western blotting membrane by comparison to parallel background readings of equal area (Quantity One, BioRad). The ratio of MMP-2 and MMP-9 to β-actin was used to evaluate protein mass as a function of SDS treatment.
Statistical analysis

The comparisons between 0.1% and 2% SDS in Figure 2 were made using a paired t-test as extractions were performed in tissues from the same patients. The comparisons in Figures 3 and 4 were made between patients with different status of labor using the Student’s t-test. Regression analysis was used to determine whether gestational age had a significant effect upon MMP protein or activity in Figures 5 and 6. In all cases the power was sufficient (>80%). Statistical significance was indicated by \( p < 0.05 \). All analyses were performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Data are expressed as mean ± SEM.

RESULTS

Effect of SDS upon protein extraction and pro-enzyme activity

Following extraction with 0–2.0% SDS, we compared the total protein with the MMP-2 protein extracted from amniochorion. Increasing concentrations of SDS resulted in nearly parallel increases in the mass of total protein and MMP-2 protein extracted (Figure 1). This MMP-2 protein corresponded to the 72 kD pro- (or latent) molecular weight band (shown in subsequent figures). The active, 68 kD form of the proteinase was very faint in Western immunoblots and was not quantitated.

Next the protein mass and pro-enzyme activity of MMP-2 and MMP-9 were compared when amnion tissue from term not in labor patients was extracted with 0.1% or 2% SDS (Figure 2). Both MMP-2 and MMP-9 protein mass were greater (\( p < 0.05 \)) following extraction with 2.0% SDS than with 0.1% SDS (Figure 2, left panel). The 92 kD pro-form of MMP-9 was the primary species detected by Western immunoblotting. Interestingly, MMP-2 pro-enzyme activity was less (\( p < 0.05 \)) following extraction with 2% SDS than with 0.1% SDS (Figure 2, right panel). There was no statistically significant difference in MMP-9 pro-enzyme activity between 0.1% SDS and 2.0% SDS extraction (Figure 2, right panel).

Therefore, because extraction with 2% SDS decreased the pro-enzyme activity of MMP-2, we explored whether MMP-2 pro-enzyme activity was directly suppressed by the higher concentration of SDS. Amnion tissue was homogenized with 0.1% SDS, and then the extract was split into two pools. The first pool was maintained in 0.1% SDS while the second was increased to 2.0% SDS (final concentration in the sample) prior to zymography. There was no change in the MMP-2 pro-enzyme activity due to post-homogenization SDS addition (data not shown), suggesting that the loss of relative MMP-2 pro-enzyme activity with 2% SDS extraction may have occurred during the extraction process.

We were concerned that relative changes in enzyme activity might be masked by this extraction protocol, so we next compared MMP-2 protein and pro-enzyme levels in amnion tissue from preterm and term patients following vaginal delivery and term not in labor patients and term in labor patients (Figure 3). Both MMP-2 protein levels and pro-enzyme levels increased similarly (data reported below in Results) indicating that 2% SDS extraction was an appropriate procedure for comparing relative changes in tissue MMP protein and pro-enzyme activity levels. We therefore used 2% SDS extraction to explore the changes in MMP-2 and -9 proteins and activity at term and preterm and throughout gestation in human fetal membranes.

MMP-2 and MMP-9 pro-enzyme activity and protein in the amnion at term and preterm

Zymographic analysis showed significant increases (\( P < 0.01 \), Figure 3, top left panel) in the pro-enzyme activity of MMP-2 and MMP-9 at term labor compared to term not in labor. Although there was a trend in this same direction with MMP-2 protein, neither MMP-2 nor MMP-9 protein was significantly changed between term not in labor and term in labor amnion (Figure 3, top right panel). Pro-enzyme activity and protein of MMP-2 in the amnion were higher (\( p < 0.01 \), Figure 3, bottom panels) at term labor than at preterm labor. This was
exclusive for MMP-2 as there were no such changes in MMP-9 activity or protein mass (Figure 3, bottom panels).

**MMP-2 and MMP-9 pro-enzyme activity and protein in the chorion at term and preterm**

Zymographic and Western blot analyses showed that there were no significant differences in MMP-2 and MMP-9 pro-enzyme activity and protein mass in the chorion with the onset of term labor (Figure 4, top panels) or in MMP-2 pro-enzyme activity and protein levels between preterm labor and term labor samples (Figure 4, bottom panels). However, there was less (p < 0.01) MMP-9 protein at term labor than with preterm labor, although this was not reflected to the same extent in the pro-enzyme activity.

Because we observed several changes in MMP activity and protein mass between preterm and term labor in amnion, we examined more completely the gestational age-dependent changes of non-labored and labored tissues in amnion and chorion.

**Amnion and chorion MMP-2 and MMP-9 pro-enzyme activity and protein during gestation and at term**

There was a trend for MMP-2 pro-enzyme activity to increase in amnion from non-labored patients at term, but this did not reach statistical significance (p = 0.056, Figure 5A). However, a significant increase in MMP-2 pro-enzyme activity occurred in labored patients during late gestation (y = 0.021x – 0.509,
These pro-enzyme activity increases were mimicked by increases in MMP-2 protein that were low until term when they increased (non-labored: $y = -6879 + 608x - 17.9x^2 + 0.17x^3$, $p = 0.02$; labored: $y = -2055 + 196x - 6.16x^2 + 0.06x^3$, $p = 0.003$, Figure 5B).

There were no changes in MMP-9 pro-enzyme activity in either patient group during gestation (Figure 5C), nor were there any changes in the MMP-9 protein mass in either patient group during late gestation (Figure 5D).

In the chorion, there were no changes in MMP-2 enzymatic activity in either patient group during gestation (Figure 6A), and MMP-2 protein actually decreased in non-labored patients ($y = -0.075x + 3.547$, $p < 0.05$, Figure 6B) during the third trimester. MMP-9 pro-enzyme activity did not change during late gestation (Figure 6C), but its protein mass also decreased significantly in both non-labored ($y = -0.039x + 2.613$, $p < 0.01$, Figure 6D) and labored samples ($y = -79.8 + 7.15x - 0.21x^2 + 0.002x^3$, $p = 0.011$, Figure 6D).

**DISCUSSION**

This study is the first to report that a higher concentration of SDS in the extraction buffer increases the total and MMP-2 protein and the MMP-9 protein extracted from human intrauterine tissues. This information should prove valuable for future studies of MMP roles in the human fetal membranes. Further, we report for the first time the gestational age-dependent increases in MMP-2 pro-enzyme activity and protein from labored amnion. Also, we confirm one study that MMP-2 pro-enzyme activity increases with the onset of labor at term in amnion, which suggests an induction of enzyme expression late in gestation and at labor,
contrary to many other studies, and also that MMP-9 pro-
enzyme activity increases at term labor. Finally, we dem-
strate that these patterns are different than the activity and
protein patterns of MMPs in the chorion.

Our extraction protocol was motivated by the Yu and
Woessner [10] report that rat myometrial MMP-7 is anchored
to the cell surface or the extracellular matrix, likely to heparin
sulfate proteoglycans via their hemopexin domains, thereby
making its complete extraction more difficult. These inves-
tigators emphasized that the anchoring of MMPs and their
endogenous inhibitors affect their spatiotemporal attitude
leading to activation, inactivation, or their storage in reserve.

Activation of MMP-2 begins first by activation of MT1-
MMP, a membrane-bound protease, by other proteases such as
furin (when MT1-MMP is intracellular in the Golgi) or
plasmin (when MT1-MMP is on the plasma membrane). The
activated MT1-MMP then binds the N-terminal inhibitory
domain of TIMP-2, the tissue inhibitor of MMP-2. This

membrane complex serves as a receptor for pro-MMP-2,
which when bound in this configuration, can be activated by
another molecule of MT1-MMP. The activated MMP-2
either remains bound to the TIMP-2–MT1-MMP-membrane
complex or is released [12]. We observed by Western
immunoblotting that the majority of MMP-2 protein was
evident in the 72-kD pro-MMP-2 form, which confirmed that
it would be bound to the cell membrane. However, this
anchoring might also impede extraction for subsequent protein
mass or enzyme activity analysis, and since most investigations
used low concentrations of SDS for extraction of MMPs from
human intrauterine tissues [6,13], we considered the possibil-
ity that higher concentrations of SDS might release more
MMP protein from cell surfaces or extracellular matrix
adherences.

Western blot analysis demonstrated that 2.0% SDS in the
extraction buffer is more effective in extracting MMP-2 and
MMP-9 than 0.1% SDS. But this extraction lowered the

Figure 4. MMP-2 and MMP-9 pro-enzyme activity (A and C) and protein levels (B and D) in chorion at preterm labor (PTL, n = 6), term not in labor (TNIL, n = 8), and term labor (TL, n = 8). Pro-enzyme activity and protein levels were determined by gelatin zymography and Western immunoblotting in comparison to β-actin mass (described as OD Units Ratio), respectively. Each lane in the Western blots and zymographic blots represents one patient. The pro-enzyme activity of MMPs is shown in the left-side panels (A and C) and the protein mass ratio in the right-side figures (B and D). The upper panels compare MMPs at TNIL and at TL, and the bottom panels display MMPs at PTL and at TL. Significant differences compared to PTL or TNIL are denoted by ** (P < 0.01). A significant decrease in MMP-9 protein was observed at term labor compared to preterm labor.
MMP-2 pro-enzyme activity, but not the MMP-9 pro-enzyme activity, as evident by zymography. The reasons for this are uncertain, but it is possible that the higher level of SDS in the extraction buffer may have unfolded the latent enzyme structure and caused partial enzyme inactivation by other factors in the extract. Or, the increased SDS may have had differential effects on co-factors or inhibitors that alter MMP activities during extraction. For instance, MMP-2 autolytic activation is enhanced when it is complexed with heparin. Extraction with increasing levels of NaCl, which can separate heparin from MMP-2, decreases its activity at concentrations as low as 0.1 M NaCl [14]. On the other hand, extracting MMP-2 with 0.24 M NaCl, but not 0.18 M NaCl, also extracts TIMP-1, an inhibitor of MMP-9 and, to a lesser extent, MMP-2 [15]. Because our purpose was to test a new extraction procedure for MMPs in human fetal membranes and whether new patterns of MMP levels were evident, we did not test our extracts for TIMP activities. Fortunately, the relative activity differences during gestation or with labor-dependent changes in activity were preserved regardless of SDS concentration. Importantly, when placed in context with the changing nature of gestation, the 2% SDS extraction reveals new information about tissue MMP-2 and MMP-9 levels and pro-enzyme activity in amnion. These MMPs may have a more integral role in term labor than earlier studies indicated.

MMP-9 protein levels and pro-enzyme activity in amnion or amniotic fluid are consistently elevated at term and preterm from amnion or amnion—chorion tissues or explants in other studies [4,5,8,9,16,17], and our data are in support of these observations. But our results are inconsistent with most of the literature on MMP-2 in fetal membranes. No increases in MMP-2 tissue protein or activity levels were observed with the onset of labor at term or preterm from amnion or chorion tissues combined [9], and for MMP-2 released into medium from amnion explants, there was a decrease at term labor compared to term without contractions [17]. This may have been due to lower levels of detergent in the extraction buffer and less extracted MMP-2 or to the fact that amnion and chorion have different patterns of releasing MMPs, as our study has shown, and combining them may have canceled out their individual patterns. Also, amniotic fluid levels of MMP-2 protein levels did not increase from midgestation to term [7], and indeed actually decreased [18]. However, only one study demonstrated an approximate 8-fold increase in MMP-2 zymographic activity from amnion tissue at the site of rupture with the onset of labor at term [8]. There was no detailed examination in this study of MMP-2 activity during gestation, at preterm labor or in comparison to the chorion, nor was MMP-2 protein examined at any time in this study [8]. Thus, until our current study, the majority of the data would have supported either no change or a decrease in MMP-2 levels in amnion with term labor onset.

As mentioned, there were no remarkable changes in chorion MMP pro-enzyme activity or protein levels with labor onset at
term, and these activity data confirm the MMP-2 data from chorion explants of Goldman et al. [17]. The decreasing MMP protein levels in chorion at term suggest less synthesis or greater metabolism. The most striking aspect of chorion MMP patterns is that they are considerably different from those of the amnion.

These results regarding MMP regulation and action in amnion would suggest that MMP-2 activity is induced in this tissue along with that of MMP-9, and in the case of MMP-2, this occurs in late gestation in a fashion very similar to that of prostaglandin endoperoxide H synthase (PGHS) activity [19]. Many factors have been demonstrated to induce or are associated with enhanced MMP-9 expression in reproductive tissues, including relaxin H1 and H2 [20], the integrin, alpha(v)beta(6) [21], PGE2 [22] and PGF2a [23], and several cytokines have been identified as stimulating both MMP-2 and MMP-9 in trophoblastic tissue, including TNF-α, IL-1, IL-6, and the insulin-like growth factor binding protein (IGFBP)-1 [24]. Since chorion tissue produces TNF-α, IL-1ß and IL-6 upon stimulation with lipopolysaccharide (LPS) [25], the intriguing possibility that amnion MMP-2 is induced by these or other locally produced factors remains to be demonstrated.

In conclusion, our studies demonstrate that MMP-2 and -9 pro-enzyme activities increase in amnion but not in chorion with the onset of term labor. Enzyme expression, activation and release from tissue binding sites may be key regulatory events in membrane rupture and other labor-associated mechanisms at term.

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