Evidence of in vitro differential secretion of 72 and 92 kDa type IV collagenases after selective exposure to lipopolysaccharide in human fetal membranes

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Introduction
Premature rupture of membranes (PROM) is defined as the spontaneous rupture of the fetal membranes prior to the onset of labor; it is associated with ~40–60% of all preterm births (Newton, 2005) and complicates 10% of all pregnancies (Parry and Strauss, 1998). The etiology of preterm birth (<37 weeks of gestation) is complex, but evidence indicates that infection/inflammation is involved, especially in spontaneous preterm labor (Gomez et al., 1997; Hagberg et al., 2005); in this context, intrauterine infection is the main factor associated with the development of PROM (Asrat, 2001). Clinical and epidemiological evidence supports the presence of an infectious process in the cervico-vaginal region as a plausible explanation to the following ascending intrauterine/intra-amniotic infection (Romero et al., 1988a).

During pregnancy, the presence of pathogenic microorganisms in the reproductive tract induces a proinflammatory network of signals that coordinates the defense; however, this also results in a selective collateral damage that involves the chorioamniotic membranes. Some of these signals include synthesis and secretion of proinflammatory factors and extracellular matrix (ECM)-degrading enzymes (Vadillo-Ortega et al., 2002; Zaga et al., 2004).

Fetal membranes are complex multilaminated tissues, constituted by the amnion (AMN) and chorion, two closely adherent layers consisting of several cell types, including epithelial cells, mesenchymal cells and cytotrophoblasts inter-digitized with the maternal decidua (Hampson et al., 1997; Parry and Strauss, 1998). The tensile strength guarantees their function and structure, which depend on the integrity of these cells and their associated ECM, a reticular structure constituted by collagen type I, III, IV and V, as well as laminin, fibronectin, nidogen and proteoglycans (Malak et al., 1993, 1994; Bell and Malak, 1997).

Keywords: intrauterine infection/chorioamniotic membranes/matrix metalloproteinases/extracellular matrix
Support of fetal growth and movement, protection and maintenance of the immunological and endocrinological privilege are functions of fetal membranes and are provided among other factors by the ECM, which normally remains intact until the final stages of labor. Evidence indicates that intensive disruption of ECM constituents is associated with rupture of fetal membranes both in spontaneous and premature labor (Vadillo-Ortega et al., 1990, 1995).

Most of the ECM and basement membrane components can be degraded by matrix metalloproteinases (MMP), a group of structurally related zinc-dependent enzymes. These include MMP-2 [72 kDa type IV collagenase (gelatinase A)] and MMP-9 [92 kDa type IV collagenase (gelatinase B)] which have been ascribed roles as central mediators in degradation/damage of the ECM of fetal membranes in normal and pathological conditions (Bryant-Greenwood and Yamamoto, 1995; Lei et al., 1995; Vadillo-Ortega et al., 1995, 1996). The main substrate of these enzymes is type IV collagen, which is present as a three-dimensional lattice with proteoglycans that help stabilize the type I interstitial collagen fibers. Type IV collagen is one of the principal components of the AMN and chorion and it is located both in the basal membranes and in the interstitium (Halaburt et al., 1989; Vadillo-Ortega et al., 1995).

The pathophysiology of spontaneous PROM also indicates the importance of ECM remodeling by these enzymes in the inter-layer connective tissue of fetal membranes (Gomez et al., 1997). The equilibrium between the collagenolytic activity of MMPs and their endogenous tissue inhibitor (TIMP) can be unbalanced in the presence of infection and other conditions known to be risk factors for PROM (Locksmith et al., 1999; Fortunato et al., 1999, 2000).

Some of the stages of intrauterine infection by gram-negative microorganisms can be emulated in vitro with lipopolysaccharide (LPS), a soluble component of the cell wall of these pathogens, responsible for their toxic and pathogenic effects. In different experimental models, LPS has been known to cause fetal death or abortion and a robust proinflammatory response in different animal models and has been implicated as a cause of preterm labor in humans (Cox et al., 1988; Romero et al., 1988b; Silver et al., 1995; Grigsby et al., 2003).

In this study, we employed an ex vivo model that allowed us to reproduce the differential contact between the AMN/CHD region with the LPS and to know the source and secretion pattern of MMP-2, MMP-9, TIMP-1, TIMP-2 and TIMP-4 on both sides of the membranes after selective stimulation with this compound.

Materials and Methods

Fetal membrane explants culture

After obtaining their written informed consent, women at 37–40 weeks of gestation without evidence of active labor and with neither clinical nor microbiological signs of chorioamnionitis nor of lower genital tract infection were included in the protocol. This project was approved by the Internal Review Board of the Instituto Nacional de Perinatología ‘Isidro Espinosa de Los Reyes’ in Mexico City (Registry No. 212250-06101).

Eleven fetal membranes were obtained after delivery by elective Cesarean section, cut at a distance of 5–6 cm from the placental disc, transported to the laboratory in sterile Dulbecco Modified Eagle Medium (DMEM; Gibco BRL, Bethesda, MD, USA), and rinsed in sterile Hanks balanced salt solution (Gibco BRL) to remove adherent blood clots. Segments representing all zones of the membranes were manually cut into 18 mm diameter discs and held together with silicone rubber rings to be placed on the upper chamber of a Transwell system (Costar, New York, NY, USA) from which the original polycarbonate membrane had been removed previously. In this model, the chorio-decidual (CHD) faces the upper chamber of the Transwell system and the AMN faces the lower chamber, this allows for the testing of the two independent compartments delimited by the membrane. A detailed description and validation of this model has been published previously (Zaga et al., 2004). One milliliter of DMEM supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate and 1X antibiotic-antimycotic solution (penicillin 100 U/mL, streptomycin 100 μg/mL) (DMEM-FCS) (Gibco BRL) were added to each chamber. The mounted explants were placed in a 12-well tissue culture plate (Costar) and incubated in 5% CO2 at 37°C.

Explant stimulation

Chorioamniotic membrane explants were pre-incubated for 48 h in the medium (DMEM-FCS) to stabilize tissues after manipulation. Subsequently, the explants were washed with saline solution to remove FCS and the remainder of the medium was changed to DMEM with 0.2% lactalbumin hydrolysate (Gibco BRL), and co-incubated with 500 ng/ml LPS (from Escherichia coli 055:B5) (Sigma, St Louis, USA); this concentration has previously been shown to be effective to induce a significant increase of TNFα and IL-1β secretion in chorioamniotic membranes in vitro (Arechavaleta-Velasco et al., 2002; Zaga et al., 2004).

Each experiment included the following set of chambers, in triplicate: Basal, control membranes in which only the medium was added to the compartments; Both, LPS was added simultaneously to the CHD and AMN compartments; CHD, LPS was only added to the CHD side; AMN, LPS was only added to the compartment in contact with the AMN. The medium from either AMN or CHD chambers was collected after 24 h of incubation and stored at −70°C until assayed. Protein concentration in all samples was assessed with the Bradford method (1976).

Structural analysis

After stimulation, membrane fragments of 0.25–0.3 cm² were fixed in Karnovsky buffer, pH 7.3 (0.1 M sodium cacodylate, 2.5% glutaraldehyde) (Sigma Chemical Co, St Louis, MO, USA) for 2 h at room temperature. Samples were dehydrated by standard techniques and included in Epon (Pelco, Glovis, CA, USA). Slides with 0.9–1.0 μm sections were stained with 0.5% toluidine blue (Sigma) and analyzed through light microscopy.

Zymography

SDS-polyacrylamide gels (8%) co-polymerized with porcine gelatin (1 mg/ml) containing gelatin were prepared according to the standard methods previously described by Woessner et al. (1995). All samples were normalized to total protein concentration, and equal amounts of protein were loaded (0.5 μg) under non-denaturing conditions and run under a constant current (10 mA) for 1.6 h. Gels were washed in 2.5% Triton X-100 for 30 min to eliminate SDS remnants and incubated overnight at 37°C in a buffer of 50 mM Tris, pH 7.4, 0.15 M NaCl, 20 mM CaCl₂ and 0.02% NaN₃. Gels were stained with 0.1% R-250 brilliant blue (Boehringer Manheim, IN, USA). A standard for MMP-2 and MMP-9 activity obtained from U-937 promyelocyte cells medium (ATCC; Rockville, MD, USA) was included in each gel. Concentrations of protein loaded onto the gels were analyzed within linearity of enzymatic activity; quantitation and comparison of the gelatinolytic activity (relative intensity of lysis bands) of MMP-9 and MMP-2 were performed by densitometric analysis with the NIH-Image software program V 1.6 (NIH, Bethesda, USA).

MMP-2 and MMP-9 enzyme-linked immunosorbent assay

A commercial kit (Amersham Biosciences, Buckinghamshire, UK) was used for quantification of pro-enzymatic and active forms of both gelatinases, which allows for the specific determination of both forms of MMP-9 and MMP-2 (Hanemaaijer et al., 1999; Edwin et al., 2002). The experimental procedures were done according to the manufacturers’ instructions. Briefly, both systems are based on the detection of endogenous active MMP-2 or MMP-9; quantitation for total enzyme (active and zymogen) was done by artificial activation with APMA (p-aminophenylmercuric acetate). Both MMP-9 and MMP-2 are secreted to the extracellular compartment and can be detected in the culture medium. Proteins were captured onto the wells by incubating at 4°C, for 18 h, in a 96 well plate coated with a specific antibody. After washing, the plates were incubated at 37°C with the substrate protein and, after 4 h, the resulting reaction was read at 405 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. MMP-9 was measured in the
range of 0.5–16 ng/ml with a sensitivity of 0.5 ng/ml. The MMP-2 standard curve was 0.75–12 ng/ml with a sensitivity of 0.5 ng/ml.

**TIMP-1, TIMP-2 and TIMP-4 enzyme-linked immunosorbent assay**

Commercial kits were used for the quantitative determination of TIMP-1, TIMP-2 (Amersham Biosciences, Buckinghamshire, UK) and TIMP-4 (R&D Systems, Minneapolis, USA). Experimental procedures were done according to the manufacturers’ instructions. The assays are based on a two-site ELISA sandwich format, which uses two antibodies directed against different epitopes. Samples and standards were incubated in duplicate in the plates precoated with specific antibodies for 2 h at room temperature; after this time, plates were washed and incubated with horse-radish peroxidase-conjugated antibody to form an immobilized complex. The resulting color was read at 405 nm for TIMP-1 and TIMP-2 and at 450 nm for TIMP-4.

TIMP-1 was measured in the range of 3.13–50 ng/ml with a sensitivity of 1.25 ng/ml; TIMP-2 standard curve was 8–18 ng/ml with a sensitivity of 3 ng/ml and TIMP-4 standard curve was 0.078–5 ng/ml with a sensitivity of 0.002 ng/ml.

**Immunohistochemistry**

After stimulation with LPS, sections of 10–15 μm of paraffin-embedded chorioamniotic membranes were processed for immunohistochemistry with the avidin-biotinylated peroxidase complex technique (Vector, Burlingame, CA, USA) as described previously (Vadillo-Ortega et al., 1995).

The same membranes without stimulation were used as control. Monoclonal antibodies against MMP-9 [Ab-3, Cat No. IM37L] or MMP-2 [b-3, Cat. No. IM33L] (Calbiochem Darmstadt, Germany) were used for the immunolocalization of active and latent isoforms. Monoclonal antibodies were pre-absorbed with pure MMP-9 or MMP-2 and used as a negative-staining control to demonstrate antibody specificity in each group of slides. Three independent blinded observers evaluated all sections.

**Tissue extract**

After stimulation with LPS, the membranes were washed three times with cold PBS; subsequently, the tissue was exposed to a thermal shock (4°C for 10 min, 60°C for 3 min, −20°C for 10 min and 37°C for 5 min) in buffer A (50 mMTris Base, 100 mM NaCl, 20 mM CaCl₂, 0.02% NaN₃ and 2% Triton 100X). After these incubations, the tissue was transferred and homogenized in 1 ml of buffer B (50 mM Tris Base, 100 mM NaCl, 40 mM EDTA, 0.02% NaN₃). The soluble fraction was obtained after centrifugation at 15 200 g, 20 min; proteins were quantified by the Bradford method and 6 μg were loaded on a zymogram.

**Statistical analysis**

Comparisons between the experimental groups and the control were performed using Kruskal–Wallis one-way analysis of variance on rank tests, and P < 0.05 was considered as significant. All graphic data show the mean ± SD and data in the table correspond to the intraquartile range 50 [25–75].

**Results**

After stimulation of the human amniochorion with 500 ng/ml LPS, the secretion pattern and gelatinolytic activity of MMP-9 and MMP-2 was modified (Fig. 1a). The zymogram densitometric analysis (n = 10) showed that, independently from the side of the membrane to which LPS had been added, the CHD secreted significant higher amounts (P < 0.01) of pro-MMP-9 (Fig. 1b) and pro-MMP-2 (Fig. 1c). The contribution of the AMN was limited, this was confirmed when the stimulus was added only in this zone. Minute amounts of active

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**Figure 1:** MMP-9 (92 kDa) and MMP-2 (72 kDa) secreted to the culture medium. Representative zymogram (a) showing enzymatic activities of both enzymes after selective stimulation of chorioamniotic membranes with 500 ng/ml of LPS; each lysis band induced by proMMP-9 (b) and proMMP-2 (c) was quantified by a densitometric analysis. Equal amounts of protein were loaded (0.5 μg). Each bar represents the mean and standard deviation of eight independent experiments; significant differences between basal and stimulated condition are indicated (*P ≤ 0.05). C. Choriodecidua; A. Amnion.
forms of MMP-2 and MMP-9 could be detected in some experiments using this methodology. The ELISA assay showed that stimulation, either individually or on both sides of the membrane, was followed by a significant increase ($P < 0.01$; $n = 10$) in the concentration of proMMP-2 in the CHD side with an average increase of $2.49 \text{ ng/ml (± 0.99)}$, a 2.5-fold increase in comparison with the basal secretion, $0.99 \text{ ng/ml (± 0.20)}$. On the other hand, in the AMN, the secretion of this pro-enzyme was not significantly different after stimulation, $1.73 \text{ ng/ml (± 0.58)}$, when compared with their basal conditions, $1.68 \text{ ng/ml (± 0.59)}$ ($P = 0.36$) (Fig. 2).

The secretion of proMMP-9 was significantly increased after LPS stimulation; the CHD gave a more potent response with an average increase of $90.9 \text{ pg/ml (± 45.04)}$, which represents a 3.6-fold increase ($P < 0.01$; $n = 10$) in comparison with the basal condition, $24.7 \text{ pg/ml (± 9.3)}$. This secretion pattern was very similar to all stimulation modalities. It is interesting to note that when both regions were stimulated, the AMN also increased significantly ($P < 0.05$) proMMP-9 secretion to the medium. However, the AMN’s response to the stimulus was not significant when LPS was added only to one side of the membrane (Fig. 3).

A constant finding under all experimental conditions was that the levels of the active forms of MMP-2 and MMP-9 did not reveal significant differences (mean of $P = 0.51$) in the culture medium with respect to their corresponding basal levels.

ELISA assays indicated that TIMP-1, TIMP-2 and TIMP-4 concentrations were not significantly different ($P = 0.43, 0.56, \text{ and 0.26, respectively}$) from those found in basal conditions (Table 1).

An extensive degradation process of the different ECM layers, mainly in the compact zone of AMN, was found after LPS stimulation. When compared with control membranes under the same culture period (72 h) (Fig. 4), the membrane exposed to LPS in the CHD region developed a loss of structural continuity associated with a degradation of the reticular structure in the compact zone; it is possible to see ‘holes’ among trophoblast cells in the chorion (Fig. 5). Similar structural changes were evident when the primary contact zone was the AMN (Fig. 6).

Cells of the amniotic epithelium and trophoblasts presented a very strong reactivity to MMP-2, the signal was localized in the basal membrane of the AMN and in the intermediate region that delimits the AMN and CHD. Immunoreactivity distribution was similar to all stimulation modalities (Fig. 7).

Specific MMP-9 immunoreactivity was revealed after selective stimulation with LPS; immunohistochemistry showed that, after simultaneous stimulation, immunoreactivity was distributed uniformly along the basal membrane of the amniotic epithelium and the compact region of the AMN, the trophoblasts of the chorion were clearly positive for this enzyme. The immunoreactivity pattern was very similar to all stimulation modalities (Fig. 8).

In order to demonstrate the presence of active forms of both gelatinases, tissues were treated as described in a recently published paper (Meraz-Cruz et al., 2006); the zymogram in Fig. 9 reveals the presence and activity of the active forms of MMP-2 and MMP-9 after co-incubation with LPS. Densitometric analyses indicate that, independently from the initial zone of exposure, the endotoxin induces a significant increase in the synthesis and activity of MMP-2 and MMP-9 ($P \leq 0.05$) in both membranes.

**Discussion**

During pregnancy, the fetus has immunologic and endocrine privileges within the amniotic cavity, this space is delimited by chorionic-amniotic membranes, which, through a finely regulated turnover of
their ECM, preserve the tensile strength that guarantees their role as a selective barrier between the fetal and maternal environments.

The abnormal rupture of the membranes during pregnancy, known as PROM, is commonly identified as the main cause of preterm birth (Asrat, 2001). A growing body of evidence suggests an association between microbial invasion of the amniotic cavity and this obstetric pathology (Gomez et al., 1997).

Different evidences have suggested that intrauterine/intra-amniotic infection favors an imbalance between MMPs/TIMPs, which results in increased enzymatic activity, favoring degradation and destruction in fetal membranes of the different elements of the ECM (Fortunato et al., 1999, 2000).

The present study was undertaken to investigate the MMP-2 and MMP-9 secretion response after LPS stimulation of the CHD region (maternal side) or the AMN (fetal side) of human chorioamniotic membranes in culture. This experimental model has been validated previously and allowed us to characterize the integral response of the chorioamniotic membranes that, notwithstanding the culture conditions, retain their capacity to separate two different compartments.

During infection with gram-negative bacteria, the LPS has been implicated in the mechanism responsible for the associated pregnancy losses (Deb et al., 2004), septic shock (Vizi et al., 2001), fetal cardiac dysfunction (Rounioja et al., 2003), neurological injury in preterm infants (Dunkan et al., 2002; Huleihel et al., 2004), and preterm delivery and PROM (Romero and Mazor, 1988; Romero et al., 1988a; Huleihel et al., 2004).

The endotoxin binds to a circulating protein, lipopolysaccharide-binding protein (LBP), presenting an endotoxin monomer to CD14. The endotoxin–LBP-CD14 complex interacts with Toll-like receptor 4 (TLR 4) on inflammatory cells leading to cellular activation and stimulation of the synthesis and secretion of proinflammatory cytokines (Espinoza et al., 2002; Takeda et al., 2003) responsible for modulating the synthesis of effectors such as MMPs (Huang et al., 1998).

Table 1: In vitro secretion of TIMP-1, TIMP-2 and TIMP-4 after selective exposure with 500 ng/ml of LPS

<table>
<thead>
<tr>
<th>Zone of exposure to LPS</th>
<th>TIMP-1 (ng/mL/μg protein)</th>
<th>TIMP-2 (ng/mL/μg protein)</th>
<th>TIMP-4 (ng/mL/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choriodecida</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>61 (32–80)</td>
<td>645 (302–990)</td>
<td>1.5 (0.64–14.62)</td>
</tr>
<tr>
<td>Both</td>
<td>67 (59–102.5)</td>
<td>560 (249–3004)</td>
<td>6.3 (2.7–20.0)</td>
</tr>
<tr>
<td>Choriodecida</td>
<td>62 (42–106)</td>
<td>547 (291–1211)</td>
<td>2.5 (0.15–3.55)</td>
</tr>
<tr>
<td>Amnion</td>
<td>40 (31–67)</td>
<td>910 (475–1983)</td>
<td>7.64 (4.9–23.4)</td>
</tr>
<tr>
<td>Basal</td>
<td>47 (32–49)</td>
<td>949 (438–2281)</td>
<td>4.12 (2.8–6.8)</td>
</tr>
<tr>
<td>Both</td>
<td>47 (39–60)</td>
<td>970 (405–1540)</td>
<td>4.32 (1.83–11)</td>
</tr>
<tr>
<td>Choriodecida</td>
<td>53 (30–41)</td>
<td>1124 (428–2091)</td>
<td>2.8 (2.0–4.8)</td>
</tr>
<tr>
<td>Amnion</td>
<td>35 (21–68)</td>
<td>1110 (496–2574)</td>
<td>2.5 (0.77–3.3)</td>
</tr>
</tbody>
</table>
Previously, we demonstrated in the same model that in vitro stimulation with LPS induces a differential response in the secretion of proinflammatory cytokines by the fetal membranes mediated by a cooperative and bidirectional communication between the AMN and the CHD, which includes intermembranal cytokine trafficking and signaling between both tissues (Zaga et al., 2004).

The experimental design used herein allowed us to model three different clinical stages of intrauterine ascending infection: (i) choriodecidual infection, in which the gram-negative bacteria have reached the CHD through ascendant colonization (corresponds to CHD LPS-stimulation); (ii) intra-amniotic infection, under this circumstance the infectious stimulus has gained access to the amniotic cavity, a condition that could happen under iatrogenic introduction of microorganisms during amniocentesis (corresponds to AMN LPS-stimulation) and (iii) chorioamniotic infection, a situation in which bacteria come in contact with both sides of the membranes at late stages of colonization, which implies that bacteria have crossed the membranes, from the CHD towards the intra-amniotic cavity.

After selective LPS stimulation, the membranes showed a significant increase in the secretion level of MMP-9 and MMP-2; however, the CHD appears as the LPS-reactive tissue and contributes most of the secretion of both gelatinases after stimulation of either the AMN or the CHD. Although our experimental design did not allow us to evaluate the specific individual contribution of the decidua and chorion, there is evidence of a direct communication pathway between both regions since the CHD secretes MMP-9 and MMP-2 after stimulating the AMN.

A remarkable finding was that enzymes in the culture medium were found only in their pro-enzymatic forms which might represent a biological non-sense. We believe that this finding can be explained because the active forms, which have higher affinity for the substrate, are in close association to their substrates, i.e. type IV collagen in basement membrane and compact zone. Recently, our group demonstrated the existence of a high-molecular weight complex composed by MMP-2, MMP-3, MMP-9, and TIMP-1 and TIMP-2 that is stabilized by Ca\(^{2+}\). This complex is tightly associated to the amniochorion ECM but can be released by EDTA treatment (Mera-Cruz et al., 2006). We applied this information to our tissues and found direct evidence that the active forms of both MMP-2 and MMP-9 are present in the stimulated tissues. Further evidence to support this assumption includes the histological evidence of the presence of extensive connective tissue degradation in the AMN and chorion after LPS stimulation and the simultaneous demonstration, by immunohistochemistry, of a strong immunoreactivity of both enzymes closely associated with the ECM. The localization of the positive immunoreactivity for MMP-9 and MMP-2 in type IV collagen enriched regions, such as the basement membrane and compact layer of the AMN, is correlated with the extensive degradation of the strongest layer of the fetal membranes that supports the tensile work of these membranes.

Increased MMP-9 secretion has been associated with physiological and pathological processes, such as preterm labor, normal delivery and PROM (Athayde et al., 1998; Locksmith et al., 1999; Maymon et al., 2001). Immunoreactivity of this enzyme has been demonstrated in the amniotic fluid of women with preterm labor (Athayde et al., 1998) and...
Figure 5: Human chorioamniotic membranes stimulated in the CHD zone. The first panel shows a membrane stimulated for 24 h with 500 ng/ml of LPS (a, ×10). A detailed analysis revealed that the ECM was degraded mainly in the compact region after stimulation (b, c and d, ×40). The intermediate layer and the reticular zone do not show apparent changes; however, the ECM that surrounds trophoblasts (TB) shows some degradation zones (e, f, ×40).

Figure 6: Human chorioamniotic membranes stimulated in the AMN zone. (a) Chorioamniotic membranes (×10) stimulated with 500 ng/ml of LPS for 24 h in the amniotic epithelium, the structural disorder and the space between the AMN and CHD are evident. The compact layer shows an extensive degradation of the ECM, there is no continuity with the CHD. The fibrous skeleton was totally degraded after incubation with the endotoxin (b, c and d, ×40). In e and f, sections show a detail (×40) of trophoblasts (TB) surrounded by ECM (×40).
Figure 7: Immunoreactivity of MMP-2 in human chorioamniotic membranes. The first panel shows a non-stimulated control membrane (a, ×20). After stimulation of the chorion region (b, ×40), strong reactivity was observed in the AMN’s compact layer and basement membrane. A massive and strong immunoreactivity was detected in trophoblast cells and along the ECM of the chorion (c and d, ×40).

Figure 8: Immunoreactivity of MMP-9 in human chorioamniotic membranes. The first panel shows a non-stimulated control membrane (a, ×20). After stimulation with 500 ng/ml of LPS in the chorion region, strong reactivity was observed in the epithelium (b, ×40). Intense immunoreactivity in the basement membrane of the chorion and trophoblast cells, as well as in the extracellular space along the whole layer of trophoblasts in the chorion can be observed (c and d, ×40).

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in fetal plasma during normal labor and PROM (Riley et al., 1999). Besides, the presence of intra-amniotic infection has also been related to increased MMP-9 concentrations in the amniotic fluid and associated with a simultaneous decrease in TIMP-2 (Riley et al., 1999; Maymon et al., 2000).

On the other hand, secretion of MMP-2, a gelatinase usually signaled as a constitutive enzyme (Riley et al., 1999; Maymon et al., 2000), was increased after stimulation with LPS. During pregnancy and labor, the expression of the MMP-2 gene is constitutive; however, the process of secretion and activation of this enzyme increases significantly 24 h before the start of normal labor (Hulboy et al., 1997; Meraz-Cruz et al., 2003; Zaga-Clavellina et al., 2006). MMP-2 synthesis and secretion increase in the cases of clinical chorioamnionitis and has also been shown in an experimental model of CHD infection in pregnant rhesus monkeys (Vadillo-Ortega et al., 2002).

Fortunato et al. (1999) have indicated that LPS stimulation of chorioamnion membranes in cultures induces a significant increase in MMP-2 and MMP-9 transcription and synthesis that is associated with decreased secretion of TIMP-2, shifting the balance in favor of gelatinase activity and leading to membrane degradation that may predispose to PROM; these alterations change the stoichiometric equilibrium between enzymes and inhibitors (Fortunato et al., 1999, 2000). In contrast, our results show that this equilibrium is broken by over-secretion of MMP-9 and MMP-2 in combination with no changes in the secretion of either TIMP1 or TIMP-2, which also inclines the balance toward degradation. Similar findings were observed after stimulation of amniochorion in culture with Streptococcus agalactiae (Zaga-Clavellina et al., 2006).

Identification of MMP-2 and MMP-9 in their proenzymatic form in the culture medium after LPS stimulation simultaneously with the histological evidence of degradation of the different elements of ECM allows us to propose that the chorioamnion must contain a non-described activation mechanism for MMPs that needs to be turned on in order to render the active forms of these enzymes.

We can conclude that LPS-containing gram-negative microorganisms might interact with fetal membranes and induce expression and activation of MMP. These enzymes probably accumulate in the extracellular space before their activation as part of the pathological environment associated with the development and progression of premature rupture of the membranes driven by local infection.

Acknowledgements

We thank Jose Baltazar Garcia and Pedro Flores for performing the tissue processing methodology.

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