Effect of Experimental Genital Mycoplasmosis on Production of Matrix Metalloproteinases in Membranes and Amniotic Fluid of Sprague–Dawley Rats

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Problem
Preterm, premature rupture of membranes (PPROM) is a dire pregnancy outcome that is frequently associated with infection by the genital mycoplasmas, *Mycoplasma hominis*, *Ureaplasma parvum*, and *U. urealyticum*. One potential mechanism by which these microorganisms may cause PPROM is by increasing the concentration of matrix metalloproteinases (MMPs) in the membranes and amniotic fluid. We tested this hypothesis in a well-defined model system of genital infection with *M. pulmonis*, a natural reproductive pathogen of rats.

Method of study
Timed-pregnant, specific pathogen-free, Sprague–Dawley rats were infected with 10^7 CFU *M. pulmonis* at gestation day (gd) 14. Controls received an equivalent volume (100 μL) of sterile medium. At gd 18, rats were euthanized, and membranes and amniotic fluids were harvested and stored at −70°C until analysis. Proteinase activity of amniotic fluid and membranes was resolved on discontinuous 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gelatin zymography gels. Band intensity was determined using a digital gel documentation system and the manufacturer's software (Kodak).

Results
Gelatinolytic activity associated with a band similar in molecular weight to ProMMP-9 (92 kDa, the inactive precursor of MMP-9) was significantly increased in amniotic fluids and membranes harvested from *M. pulmonis*-treated pups at gd 18 when compared with tissues harvested from control pups. Both ProMMP-9 and ProMMP-2 (72 kDa, the inactive precursor of MMP-2) were increased in infected animals at gd 21.

Conclusion
Our study suggests that the genital mycoplasmas can increase MMP-9 production *in vivo*.

Introduction

Preterm premature rupture of membranes (PPROM) is a common pregnancy complication that may lead to chorioamnionitis, preterm labor, neonatal sepsis, and fetal death.[1] Infection is frequently associated with PPROM, and genital mycoplasmas such as *Mycoplasma hominis*, *Ureaplasma parvum*, and *U. urealyti-*

cum are often isolated from the reproductive tract of women with this condition.[2,3]

Mycoplasma hominis and *Ureaplasma* sp. are thought to cause damage to the membranes by increasing the production of proinflammatory cytokines at the maternal–fetal interface through interactions with immune cells or direct interactions with trophoblast cells themselves. Indeed, both *M. hominis* and *U. urealyticum* increased the production of tumor necrosis factor-alpha (TNF-α) and nitric oxide by murine macrophages *in vitro*,[4] and both microorganisms can cause preterm labor when administered by intra-amniotic infusion into rhesus monkeys.[5,6] TNF-α and other proinflammatory cytokines can disrupt pregnancy in a number of ways, including upregulation of the production of matrix metalloproteinases (MMPs).

Matrix metalloproteinases comprise a family of over 20 members and can be divided into groups based on their substrates (e.g. stromalysins, gelatinases, collagenases, etc). MMP-9 is particularly important in remodeling of the membranes and cervix during parturition. MMP-9 is upregulated in the membranes at the time of labor in the human, monkey, and rat.[7–9] This enzyme is also increased in the amnion by group B streptococci[8] or bacterial cell wall components such as lipopolysaccharide (LPS).[10] MMP-2 has also been shown to be increased during preterm rupture of membranes.[11] Whether genital mycoplasmas that lack LPS can increase MMP-9 or MMP-2 production in this tissue is unknown.

Our laboratory has been investigating the pathophysiology of genital mycoplasmosis using Sprague–Dawley rats and *M. pulmonis*. This animal model system has several advantages for investigations in reproductive pathology because it uses a natural pathogen of rodents that is associated with increased production of proinflammatory cytokines, low birthweight and fetal wastage.[12] In this study, we tested the hypothesis that genital mycoplasmosis causes increased production of MMP-2 and MMP-9 in the membranes and amniotic fluid.

Materials and methods

Materials

Coomassie Blue R-250 and acrylamide-bis acrylamide solution were purchased from Bio-Rad (Hercules, CA, USA). All other chemical reagents were purchased from either Sigma (St Louis, MO, USA) or Fisher Scientific (Atlanta, GA, USA). *Mycoplasma pulmonis* strain X1048 used for this project was generously provided by Dr Maureen Davidson (West Lafayette, IN, USA). A stock culture was grown in Frey's broth, stored in aliquots at −85°C, and a single vial from this stock culture was thawed just prior to use. The thawed culture contained 10^8 CFU/mL when thawed, and the CFU was confirmed for each experiment.

Animals and Treatments

All experiments were performed in accordance with University of Florida Institutional Animal Care and Use Committee-approved protocols. Timed-pregnant, specific pathogen-free (SPF) Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN, USA) and delivered to the University of Florida at gestation day (gd) 11 or 12 and maintained under SPF conditions at all times. Rats were monitored by the commercial vendor and were presumed SPF for the following: Sendai virus, H-1 virus, rat coronavirus, sialodacryoadenitis virus, reovirus type 3, Kilham rat virus, Hantaan virus, *M. pulmonis*, respiratory and enteric pathogens, endoparasites and ectoparasites and housed in Microisolator® (Lab Products, Inc., Maywood, NJ, USA) cages. In order to maintain SPF conditions, all animals were handled within a laminar airflow hood. All food, water and caging were autoclaved prior to use. Control rats were always handled first and were housed separately from inoculated rats.

On gd 14, rats were anesthetized with an intraperitoneal dose of ketamine (25 mg; Ketaject, Phoenix Pharmaceutical Inc., St Joseph, MO, USA) mixed with xylazine (0.375 mg; Xylazine-20, The Butler Co., Columbus, OH, USA) to produce 40 min of anesthesia. Animals then received either 10^7 CFU *M. pulmonis* via intracardiac injection, or an equivalent volume of sterile Frey's broth. This method results in nearly a 100% infection rate for the placenta and amniotic fluid by gd 18,[13] severe histological placentitis, and increased production of proinflammatory cytokines at the maternal–fetal interface.[13,14]

At necropsy, gd 18 or 21, rats were anesthetized with an overdose of sodium pentobarbital (180 mg; Veterinary Laboratories Inc., Lenexa, TX, USA) injected intraperitoneally. After deep anesthesia was obtained, rats were exsanguinated by transection of the femoral artery and vein. Fetal rat pups died *in utero* upon death of the dam. Samples of maternal and fetal tissues were collected to determine the

infection status of the rats and their pups. Membranes and amniotic fluid were collected from the fetal units, snap-frozen in liquid nitrogen and stored at −85°C until zymography.

Zymography of Rat Amniotic Fluid and Membranes

Amniotic fluid was thawed and clarified by centrifugation at 10,000 × *g* for 15 min at 4°C. The clarified amniotic fluid was mixed 1:3 with zymography loading buffer and loaded onto discontinuous 7.5% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels that contained 0.5 mg/mL gelatin. Amniotic fluid obtained at gd 18 was analyzed for MMP-2 (1.6 μL loaded per lane) and for MMP-9 (3.4 μL amniotic fluid loaded per lane). After electrophoresis, gels were incubated in 2.5% Triton X-100 for 1 hr and then incubated in zymography development solution for 18–20 hr at 37°C. Gels were incubated in 0.5% Coomassie Blue in 40% methanol, 7% acetic acid, 53% water R-250 for 1 hr, destained for 1 hr in 40% methanol, 7% acetic acid, 53% water, and photographed using a digital gel analysis system. With this method, bands of proteolytic activity appear as clear bands on a blue background. Band intensities at approximately 72 kDa (ProMMP-2) and 92 kDa (ProMMP-9) were quantified using the Kodak 1D digital gel analysis and documentation system (Rochester, NY, USA).

Membranes were homogenized in 5 mL lysis buffer (50 mM Tris–HCl, 2 M urea, 1 g/L Brij 35, and 0.1 mM phenylmethylsulfonyl fluoride) and the proteins were clarified by centrifugation at 15,000 × *g* for 15 min. Total protein concentration in the supernatant was measured using the Bio-Rad protein reagent with bovine serum albumin as standard. Protein (100 μg) was then precipitated by adding ice-cold acetone to 90% (v/v) and separated by centrifugation at 10,000 × *g* for 15 min at 4°C. Samples were then prepared by resuspending the proteins in loading buffer, and 6 μL (30 μg) was analyzed by zymography as described above.

Real-Time RT-PCR for MMP-9

Membranes were homogenized in 1 mL Trizol reagent (Gibco, Grand Island, NY, USA) and total RNA was extracted and precipitated according to the manufacturer's instructions. Complementary cDNA was prepared from 2 μg total RNA using a cDNA archive kit (Applied Biosystems International, Foster City, CA, USA). Real-time polymerase chain reaction (PCR) was performed in duplicate reactions on 2 μL cDNA using master-mix, PCR primers and probes for MMP-9 and 18S rRNA using the TaqMan™ system and associated software as directed by the manufacturer (Applied Biosystems, Foster City, CA, USA).

Statistics

Potential differences in ProMMP-9 and ProMMP-2 band intensity were evaluated by generalized estimating equations using the generalized linear models procedure of SAS (SAS Institute, Cary, NC, USA). Data for MMP-9 gene expression (cycles to amplification) were analyzed in a similar manner except that 18S rRNA was used as a covariate to correct for potential differences in RNA loading. Differences were considered significant at the $P < 0.05$ level, and data are shown as least-squares mean ± S.E.M.

Results

Culture results for the samples used for these analyses are shown in Table I. Level of infection was gen-

Table I Culture Data for all Samples Analyzed by Zymography for MMP-2 and MMP-9 and Real-Time Polymerase Chain Reaction for MMP-9

	Culture results[a]		
Samples (method of analysis)	Amniotic fluid (%)	Placenta (%)	Fetal spleen–liver (%)
gd 18 Amniotic fluid (zymography)	46/46 (100)	22/22 (100)	21/22 (95.4)
gd 18 Membranes (zymography)	41/41 (100)	38/38 (100)	36/38 (94.7)
gd 18 Membranes (RT-PCR)	16/16 (100)	14/14 (100)	13/14 (92.8)
gd 21 Membranes (zymography)	ND	19/20 (100)	14/24 (58.3)

ND, not done; RT-PCR, reverse transcription-polymerase chain reaction; MMP, matrix metalloproteinase; gd, gestation day.
[a]Number of positive samples/samples randomly selected for the analyses of this study (%).

erally higher for amniotic fluid and placenta than for the spleen–liver. Gelatinolytic activity was consistently observed at ~92 and ~72 kDa, corresponding to ProMMP-9 and ProMMP-2. The gelatinolytic activity of these bands could be inhibited by addition of 10 mM ethylenediaminetetraacetic acid to the development solution suggesting that the bands were MMPs (data not shown).

Significantly more activity (band intensity) was observed in amniotic fluid (Fig. 1) and membranes (Fig. 2) for the band corresponding to ProMMP-9 in animals that received *M. pulmonis* than in control animals at gd 18. Real-time reverse transcription (RT)-PCR demonstrated that fewer cycles of amplification were required for MMP-9 in the membranes collected from control animals than in tissues harvested from infected animals (Fig. 3). No significant differences were detected for gelatinolytic activity for the band corresponding to ProMMP-2 in amniotic fluid or membranes at gd 18. At gd 21, however, more activity for both ProMMP-9 and ProMMP-2 was present in membranes of *M. pulmonis*-infected dams than control dams (Fig. 4).

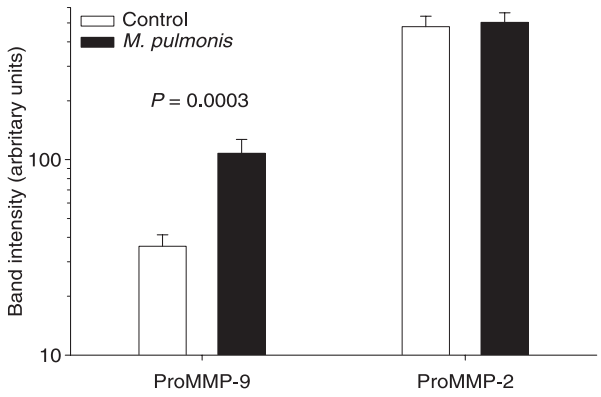

Fig. 1 Pro-matrix metalloproteinase-9 (ProMMP-9), is increased in membranes of pups at gestational age 18 that were harvested from dams infected with *Mycoplasma pulmonis* at gestation day 14. Shown are the least-squares mean ± S.E.M. for pixel intensity for each group (note that data are shown on a logarithmic scale).

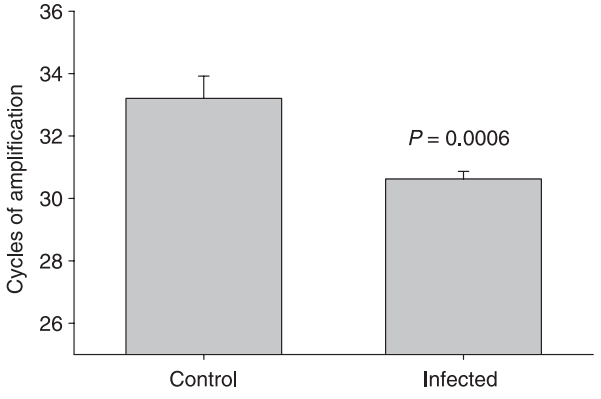

Fig. 3 mRNA for matrix metalloproteinase-9 (MMP-9) is increased in membranes of gestation day 18 pups harvested from *Mycoplasma pulmonis*-infected pregnancies. Shown is the least-squares mean ± S.E.M. of the number of cycles to threshold, which is inversely and geometrically proportional to the level of mRNA.

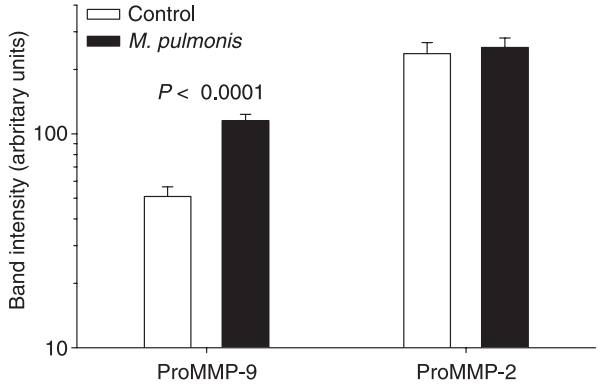

Fig. 2 Pro-matrix metalloproteinase-9 (ProMMP-9), is increased in amniotic fluid of pups at gestation day 18 from dams infected with *Mycoplasma pulmonis* at gestation day 14. Shown are the least-squares mean ± S.E.M. for pixel intensity for each group (note that data are shown on a logarithmic scale).

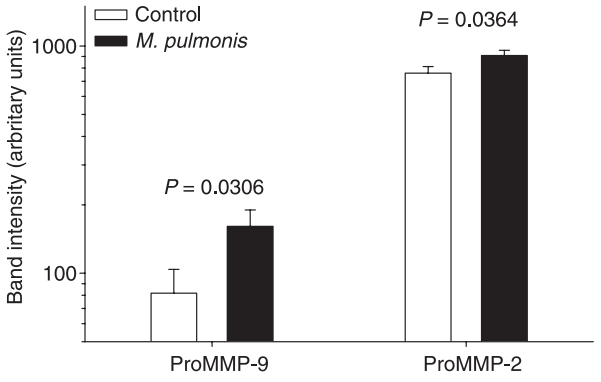

Fig. 4 ProMMP-9 and ProMMP-2 are increased in the membranes of gestation day 21 pups harvested from dams infected with *Mycoplasma pulmonis* at gestation day 14. Shown are the least-squares mean ± S.E.M. for pixel intensity for each group (note that data are shown on a logarithmic scale).

Discussion

Preterm premature rupture of membranes accompanies about 50% of all preterm deliveries and is frequently associated with infection.[15] The genital mycoplamas *M. hominis* and *Ureaplasma* sp. are most common pathogens of the reproductive tract. These organisms have been isolated from amniotic fluid harvested from women with intact membranes.[16] It has been reported that vaginal colonization or amniotic infection with this organism significantly increases the likelihood of PPROM.[2,3]

Infection with *Ureaplasma* sp. in women is associated with increased concentrations of TNF-α in amniotic fluid[16] and experimental infection of Rhesus monkeys with either *U. urealyticum* or *M. hominis* increases the production of TNF-α in amniotic fluid.[5,6] This cytokine has been shown *in vitro* to stimulate MMP-9 production by amnion[10] or amniochorion.[17] *Mycoplasma pulmonis* has previously been shown to increase TNF-α concentrations in amniotic fluid in experimentally infected rats.[12] Therefore, we hypothesized that genital mycoplasmas would increase *in vivo* production of MMP-9 in a well-defined system of intra-amniotic infection.

We found that MMP-9 was significantly increased in the membranes and amniotic fluids of *M. pulmonis*-infected dams. Although *M. pulmonis* is a different species than the genital pathogens affecting humans (*U. parvum*, *U. urealyticum*, *M. genitalium*, and *M. hominis*), there are many similarities in the pathophysiology of these organisms with regard to their consequences on infertility and fetal wastage.[12] Although no overt clinical effects of infection on pregnancy are observed with this model, intravenous injection of *M. pulmonis* into pregnant rats results in high levels of bacterial infection in amniotic fluid, placenta and endometrium as well as histological placentitis.[14] Future studies comparing the pathophysiology of infection by the intravaginal route with the intravenous route may be valuable for separating clinical from subclinical aspects of intrauterine infection. This is particularly relevant as the clinical course of genital mycoplasmosis in humans is most closely linked to clinically silent chorioamnionitis rather than febrile intrauterine infections.[18] This study suggests that increased production of proinflammatory cytokines and MMP-9 occurs during genital mycoplasmosis but this is not sufficient for induction of PPROM, preterm labor or low birthweight in this model system. During spontaneous labor in monkeys, ProMMP-9 increases as labor approaches followed by increased production of activated MMP-9 (83 kDa).[8] It is also possible that MMP-9 must be transformed to its active form in order for it to digest membrane collagen and that the enzymes which activate proMMP-9 are not increased in this model.

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