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A role for matrix metalloproteinase-9 in pathogenesis of urogenital *Chlamydia muridarum* infection in mice

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Abstract

Matrix metalloproteinases (MMPs) are a family of host-derived enzymes involved in the turnover of extracellular matrix (ECM) molecules and the processing of cytokines, chemokines and growth factors. We have previously reported that global inhibition of MMP in *Chlamydia muridarum* urogenital tract infection of susceptible strains of female mice impeded ascension of *C. muridarum* into the upper genital tract, blunted acute inflammatory responses and reduced the rate of formation of chronic disease. Because we have also observed that MMP-9 (also known as gelatinase B) is expressed in relatively large quantities in susceptible strains of mice in response to infection during acute phases of infection, we explored this further in a more selected fashion. We infected MMP-9 gene knockout mice and wild type controls intravaginally with *C. muridarum*. Both groups of mice had similar isolation rates from the lower urogenital tract but the absence of MMP-9 resulted in a slightly lower isolation rate in the upper genital tract, blunted acute inflammatory indices in the affected tissues and a reduced rate of formation of hydrosalpinx – a surrogate marker of infertility. These results imply that MMP-9 is involved in pathogenesis of chlamydial infection in this model possibly by amplifying inflammatory responses.

Keywords

CHLAMYDIA; MATRIX METALLOPROTEINASE-9; MICE; INFLAMMATION

1. INTRODUCTION

The mouse model of female urogenital tract infection with *Chlamydia muridarum* (previously, the mouse pneumonitis strain of *Chlamydia trachomatis*, MoPn) has been used for some time to study immunological protection related to chlamydial infections (1,2). More recently, it has been used to study pathological immune responses during chlamydia infection. Hydrosalpinx formation, infertility and fibrotic oviduct occlusion are routine consequences of infection following a single intravaginal inoculation of *Chlamydia muridarum* in susceptible mouse

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strains (3–7). These sequelae also serve to make the mouse:MoPn infection model a legitimate model to study chlamydia-induced host response-mediated pathology.

Matrix metalloproteinases are a class of zinc-dependent enzymes that are involved in the proteolysis and re-synthesis of the ECM (8,9), processing of chemokines and cytokines to active forms (10), the release of sequestered growth and signaling factors (11) and chemotaxis and migration of leukocytes through inflamed tissues (12–14). With regard to chlamydial infections, a role for MMP has also been proposed in trachoma (15,16) and enhanced MMP expression has been reported in an *in vitro* model of human fallopian tube infection (17). In the mouse:MoPn urogenital infection model, we have observed elevated MMP expression in susceptible strains when compared to resistant strains – especially during peak acute inflammatory responses (18). Matrix metalloproteinase-9 (MMP-9, aka gelatinase B) was particularly elevated in that study and histological studies indicated that infiltrating neutrophils was main source. Global inhibition of MMP by the administration of chemical inhibitors of their activity was shown to reduce inflammation, ascension of the infection into the upper genital tract and reduce hydrosalpinx formation (19).

Because of our previous findings, we hypothesized a role for MMP-9 in the pathogenesis of chlamydial infection in the mouse model. Mice possessing a deletion in the MMP-9 gene (MMP-9 KO) or wild type controls on a similar genetic background were infected intravaginally with MoPn and outcomes of infection course, progression to upper genital tract involvement and gross and microscopic pathology were assessed. The results confirm a role for MMP-9 in the pathogenesis of acute disease, ascension of the infection into the upper genital tract and chronic sequelae of disease in this model.

2. MATERIALS AND METHODS

2.1 Mice, infection and infection assessment

Breeder pairs of MMP-9 KO mice were a kind gift of Dr. Robert Senor of Washington University in St. Louis, Missouri and a colony was established at Northwestern University. Once the colony was established and breeder pairs confirmed as MMP-9 KO, the knockout was confirmed in subsequent breeders and in stock mice by randomly testing mice for the MMP-9 and neomycin resistance genes by polymerase chain reaction of DNA isolated from tail snips as described previously (22). All MMP-9 KO mice were between 7 and 14 weeks of age at the time of infection. Wild type (wt) B6129 were obtained from Taconic Laboratories (Germantown, New York) at 5–6 weeks of age, and allowed to acclimate for 10 days prior to beginning any experiment. The wt mice are a moderately susceptible strain of mice that reflect the F1 cross between a susceptible strain (SvEv129) and a resistant strain (C57BL/6). Susceptibility is based primarily on hydrosalpinx formation which is a surrogate marker of infertility and fibrotic oviduct occlusion (5,7,20). In our lab, the resistant strain of C57BL/6 mice routinely display a 30–40% rate of hydrosalpinx formation following MoPn infection whereas the same infection in susceptible SvEv129 results in an 80–90% rate of hydrosalpinx. The F1 generation of these two strains (B6129) from which the KO mice were derived historically has displayed a 60–70% rate of hydrosalpinx formation.

All mice were housed with rodent chow and water ad libitum with a 12:12 light:dark cycle. Mice were treated with 2.5 mg of medroxyprogesterone acetate (DepoProvera, P4, Upjohn, Kalamazoo, Michigan) and 7 days later inoculated intravaginally with 200 ID₅₀ (10⁴ inclusion-forming units, IFU) of HeLa 229-grown *C. muridarum*, MoPn (Weiss strain), exactly as described elsewhere (21). In order to verify infection, cervical-vaginal swabs were collected in all mice at day 4 post-infection and the infection course was followed in a subset of 5 mice at the specified intervals post-infection in each experiment. *C. muridarum* was isolated, inclusions visualized by indirect immunofluorescence and quantified as inclusion-forming

units (IFU) in HeLa 229 cultures as previously described (21). In order to assess upper genital tract infection, mice were euthanized, urogenital tissue above the cervix but excluding the ovary was excised, homogenized in sucrose-phosphate-glutamate buffer, sonicated, debris cleared by centrifugation and MoPn isolated as previously described (22).

2.2 Collection of tissue samples and histological assessment

In each experiment, we euthanized a subset of mice from each group on days 0, 7, 14, 21, and 56 post-infection. Based on previous observations in mice without interventions, these time points represent: immediately prior to infection (day 0); the onset of the acute inflammatory response phase in the endometrium with minimal involvement of the oviduct (day 7), peak of acute inflammation in the oviduct with some residual involvement of the endometrium (day 14), infection nadir and post-acute inflammation (day 21), and post-infection with chronic disease state (day 56) respectively (6,7). At the time of necropsy and before excision, tissues were examined *in situ* for gross macroscopic evidence of pyosalpinx or hydrosalpinx formation and any other associated abnormalities in the viscera (7). The tissues were then processed for histological assessment.

2.3 Histological assessment

Five to six excised genital tract samples at each of the time points day 0, 7, 14, 21, and 28 post-infection were preserved in buffered 10% formalin at neutral pH then embedded in paraffin and serially sectioned (5 μ m) longitudinally. Attempts were made to include cervix, both uteri, both oviducts and to include as much of the luminal structures of each tissue to the greatest extent possible in each section (AML Labs, Rosedale, Maryland). The tissues were stained with hematoxylin and eosin (H&E) (7). Each H&E stained tissue section was assessed by a board-certified pathologist (JNK) or a resident in pathology under his supervision (SRL) who was blinded to the animal numbers, treatments and groupings in the experiment with sections from uninfected mouse urogenital tract tissues included for reference to normal tissue. Histopathological assessment in at least 3 roughly serial sections of each tissue was scored exactly as described previously (19).

Some tissues at the same time points were embedded in optimum cutting temperature embedding media (OCT, Sakura Finetek, Inc., Torrance, California). Once embedded, the sections were frozen immediately at -25° C and then transferred to -70° C. Serial 5 μ m longitudinal sections were cut on a Thermo-Shandon cryotome, mounted on glass slides, fixed in methanol at -20° C for 10 minutes, rinsed in phosphate-buffered saline (PBS, pH 7.2) and blocked in 5% normal rabbit serum in PBS at room temperature for 20 minutes. Cryosections of tissues were then incubated with the primary antibody, goat anti-*C. trachomatis* (diluted 1:200 in PBS-2% rabbit serum, Fitzgerald Industries Concord, Massachusetts). Following appropriate rinses in PBS, the secondary antibody Vectastain ABC Elite for Goat IgG (Vector Labs, Burlingame, California) was diluted according to the manufacturer's protocol and incubated for 30 minutes at room temperature. The slides were then rinsed again and developed according to the manufacturer's protocol. Following PBS rinse, counterstaining with hematoxylin and dehydration, the slides were then mounted with glass coverslips utilizing Permount (Fisher Scientific, Pittsburgh, Pennsylvania). Mounted slides were viewed on a Zeiss Axiovert 25 microscope with a mounted AxioCam MRc5 digital camera and Axiovision 4.2 image capturing software (Carl Zeiss USA, Inc, Thornwood, New York). Captured images were processed in Adobe Photoshop 6.0 and exported in an appropriate file format for publication purposes.

Statistics

Infection course was compared by two-way analysis of the variance (ANOVA, treatment group, days) with repeated measures (days). The incidence of acute (pyosalpinx) and chronic

(hydrosalpinx) gross pathology was assessed using Chi-square statistic with Yates correction. The occurrence of histopathological parameters was analyzed using the Mann-Whitney U test.

3. RESULTS

3.1. MMP-9 gene deletion has no effect on shedding of MoPn from the lower urogenital tract but blunts ascending infection into the upper genital tract

Fig. 1, panel A shows the effect of MMP-9 gene deletion on the course of chlamydial infection as assessed by quantity of IFU isolated from the lower urogenital tract. There were no discernable differences in the course of infection in 3 separate experiments and assessing a total of 15 mice in the MMP-9 KO and wt groups. However, Fig. 1B shows the magnitude of the infection in the upper genital tract was significantly reduced in the MMP-9 KO group ($2.5 \times 10^5 \pm 2.9 \times 10^4$ IFU) when compared with the wt mice ($7.9 \times 10^5 \pm 2.5 \times 10^4$ IFU) at day 7 post-infection ($P < 0.002$, two-tailed t test). By day 14, the difference between the MMP-9 KO ($6.5 \times 10^4 \pm 2.5 \times 10^4$ IFU per tissue) and wt ($9.8 \times 10^4 \pm 2.5 \times 10^4$ IFU per tissue) groups did not quite achieve significance ($P < 0.08$). We also utilized immunohistochemistry to stain frozen sections of upper genital tract tissue for chlamydial antigen to discern differences in upper genital tract involvement of the infection between the two groups. However, likely due to the insensitivity of this method, the differences were not readily discernable. These data imply no alteration in the shedding of viable MoPn from the lower urogenital tract and a slight but statistically significant reduction in the magnitude of infection in the upper genital tract at earlier time points post-infection.

3.2. MMP-9 gene deletion results in significantly reduced acute and chronic gross pathological outcomes

Panel A and B of Fig. 2 represent combined data from three separate experiments. Panel A shows that the incidence of macroscopically evident pyosalpinx was significantly lower in MMP-9 KO mice at day 14 (38%) than wt mice (60%) at the same time point. Also at 21 days post-infection the incidence of pyosalpinx was reduced from 43% to 20%. These data indicate a blunting of the inflammatory responses in the MMP-9 KO mice. With regard to chronic disease, Panel B of Fig. 2 shows that MMP-9 gene deletion correlates well with protection. A reduction in hydrosalpinx formation was observed in the MMP-9 KO mice (31%) when compared with the wt mice (68%).

3.3. MMP-9 gene deletion results in altered inflammatory indices in the female mouse urogenital tract in response to *C. muridarum* infection

In order to better discern the protective effect of the MMP-9 gene deletion as it relates to the pathological outcomes observed in Figure 2, we assessed H&E stained histological sections of specific regions of urogenital tissues from infected MMP-9 KO and wt mice. Fig. 3 shows the composite results of histopathology scores from 2 experiments, 6 to 8 mice per time point and at least 3 sections per mouse. When compared with wt controls, MMP-9 KO mice had reduced acute inflammatory indices (defined as polymorphonuclear granulocytes, predominantly neutrophilic infiltrates) in the endocervix (day 14 and 21) and oviduct (day 7 and 14). However, while acute inflammation appeared to be resolving in the ectocervix and endocervix in the wt mice, it remained elevated in the MMP-9 KO mice at day 21. Chronic inflammatory indices (predominantly mononuclear leukocytes) in the endocervix and uterus were reduced as a result of the MMP-9 gene deletion at day 14 but were significantly elevated in the oviduct.

While these data largely agree with the gross histology results for pyosalpinx shown in Fig. 2, Fig. 4 shows an interesting observation that was noted on many of the MMP-9 KO mice. The MMP-9 KO mice tended to display a disproportionate degree of peritubal inflammation with

acute infiltrates surrounding the serosal surfaces of the oviducts and uterus but relatively little lumen-filling infiltrates compared with wt mice at the same time frame post-infection. This implies that MMP-9 may be involved in penetration, migration or chemotaxis of inflammatory cells through the stromal layers to reach the luminal aspect of these tissues.

4. DISCUSSION

Herein we have demonstrated a role for MMP-9 in the pathogenesis of chronic disease in a murine model of human urogenital chlamydial infection. Mice with a specific deletion in the MMP-9 gene were found to have a relative protection from hydrosalpinx formation which is associated with tubal factor infertility in the mouse model we employed. One might argue that the differences observed would be more dramatic if the MMP-9 knockout were on a more susceptible background strain such as a C3H/HeN mouse. Nonetheless, loss of MMP-9 had no effect on shedding of chlamydia from the lower genital tract and only a slight effect on ascending infection into the upper genital tract.

Even though we have found that MMP-9 is produced in abundance during infection in this model (18) and now show that the absence of MMP-9 provides relative protection, the pathological effect of MMP-9 expression is neither absolute nor exclusive. We believe that there are likely other MMP or non-MMP effectors of host-mediated damage in this model. This assertion is supported by our observations in a separate study where mice were treated with a global inhibitor of MMP activity, inflammation was dramatically blunted, ascending infection was halted and chronic disease manifestations were nearly completely ablated (19). This would imply other MMPs may also be involved. Another consideration of our present results is the possibility of compensatory up-regulation of other MMPs (e.g. MMP-2 or gelatinase A) in the absence of MMP-9 activity as has been shown in other inflammatory states (23,24). In retrospect, it would have been interesting to assess MMP-2 activity in the MMP-9 KO mice. In addition, we have shown that oxygen radical-mediated damage is also involved although this effect may also be a result of MMP-mediated activity as well (25,26). Thus, while MMP-9 is likely involved in pathological host processes involved in the initiation and possibly the evolution of chronic disease manifestations, it is not the sole player in this regard.

To understand the immunopathological role for MMP-9 in this infection one must examine the substrates for this enzyme. MMP-9 (aka, gelatinase B) degrades denatured or nonfibrillated collagens such as type IV collagen which is a constituent of the basal lamina to which columnar epithelial cells are anchored. It is not hard to imagine that disruption of the basal lamina during infiltration with inflammatory cells and the elaboration of MMP-9 could lead to sloughing of the epithelium, as has been described during upper genital tract infection in this model (7,18). However, MMP-9 has been shown to have other effects on inflammation through amplifying and modulating chemokine and cytokine activity and providing for a positive feedback loop for inflammatory responses (27,28). It would also follow that MMP-9 and other MMP could serve to assist infiltrating inflammatory cells with regard to diapedesis, chemotaxis and migration through tissues (12–14). In this way, the elaboration of MMP by inflammatory cells could function by paving a path through the extracellular matrix. Our results from histological sections showing accumulations of inflammatory cells on the serosal surface of tissues of MMP-9 KO mice but with relative sparing of the luminal aspects of upper genital tract tissues seem to substantiate this as a possibility. Lastly, it has long been known that excessive ECM degradation during an inflammatory insult can lead to concomitantly excessive ECM accumulation or fibrosis in the repair phase. Hence, fibrosis as indicated by hydrosalpinx formation, could be the exaggerated response of the host as a result of excessive MMP activity in the acute phase of the host response during infection in this model.

In summary, our results indicate a pathogenic role for MMP-9 in the mouse:MoPn model of urogenital tract infection. It will be interesting to discern the precise role or roles of MMP-9 and other MMP as well non-MMP effectors in this model.

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Figure 1. Effect of MMP-9 KO on the course of urogenital MoPn infection

Panel A depicts the course of infection in MMP-9 KO mice (filled circles) or wt mice (filled squares). Each point represents the mean IFU and standard deviation isolated from cervical-vaginal swabs of at least 15 mice over the course of 3 experiments. **Panel B** is the course of upper genital tract infection (MoPn isolated from tissue homogenates of uterus and oviduct). Asterisk indicates significant difference ($P < 0.002$).

Figure 2. MMP-9 reduces pyosalpinx and hydrosalpinx formation

Panel A represents the formation of pyosalpinx as evident on gross pathological examination in MMP-9 KO mice (white bars) and untreated mice (black bars) at days 14, and 21 post-infection. Ratios above the bars are the total number of oviducts with pyosalpinx over the total number assessed. A significant difference was seen between the two groups at day 14 and 21 post-infection (Chi-square test with Yates correction, 3 experiments). **Panel B** represents the formation of hydrosalpinx as evident on gross pathological examination in MMP-9 KO mice (white bars) or wt mice (black bars) at day 56 post-infection. Ratios above the bars are the total number of hydrosalpinges observed over the total number of oviducts assessed. MMP-9 gene deletion resulted in a statistically significant reduction in hydrosalpinx at day 56 (Chi square test with Yates correction, 3 experiments).

Figure 3. Histopathological assessment of the urogenital tract during MoPn infection in MMP-9 KO and wt mice

The bar graphs in each panel depict the mean pathological scores for MMP-9 KO mice (white bars) and wt controls (black bars) relative to uninfected tissues. Each panel column is labeled according to the tissue (ectocervix, endocervix, uterus and oviduct). The top row of panels represents scoring for acute inflammation (polymorphonuclear leukocytes). The bottom row of panels represents scoring for chronic inflammation (mononuclear leukocytes). Asterisks (*) denote statistical significance by Chi-square statistic with Yates correction (P values varied but in each case was labeled if < 0.05).

Figure 4. Histological examination of tissues scored as displaying pyosalpinx in MMP-9 KO and wt mice

Panel A is a cross section of the oviduct of a normal, uninfected mouse in parallel with infected wt and MMP-9 KO mice. **Panel B** is a similar cross section through the oviduct of wt mouse with pyosalpinx at day 14 post-infection. A significant serositis, peritubal abscess and luminal filling infiltrates are present. The plicae are blunted and epithelial erosion is evident. **Panel C** is a cross section of an oviduct of a MMP-9 KO mouse at day 14 post-infection. Significant serositis in the distal uterus (asterisk in lower portion of panel) as well as peritubal abscess is present but the lumen of the oviduct is patent and plicae are intact. All sections were cut at 5

μM, stained with H&E and are at 100X original magnification. White asterisks show area of predominantly acute inflammation.