Cryptococcus neoformans pulmonary granuloma formation is associated with matrix metalloproteinase-2 expression

S. M. MAJKAt, J. KASIMOS†, L. IZZOt & A. A. IZZOt
*Children’s Nutrition Research Center, Baylor College of Medicine, Houston, Texas, USA; †Department of Pathology and ‡Department of Microbiology, Midwestern University, Chicago College of Osteopathic Medicine, Downers Grove, Illinois, USA

The aim of this study was to investigate matrix metalloproteinase (MMP) expression during the immune response to pulmonary Cryptococcus neoformans (Cne) infection. The immune response generated in C.B-17 and C57BL/6 mice to pulmonary Cne infection has previously been characterized as type 1 and type 2, respectively, differing in the cytokines produced and leukocytes recruited during infection, influencing the extent of Cne clearance from the lung. The focus of this study was to examine changes in expression of MMP-2 and tissue inhibitor of metalloproteinase (TIMP)-2 in the lungs of Cne-infected mice during the two types (type 1 vs. type 2) of responses. C.B-17 mice that formed well-defined granulomas had elevated levels of pulmonary MMP-2 mRNA early during infection. C57BL/6 mice that had poorly defined cellular aggregates did not express detectable levels of pulmonary MMP-2 mRNA until later in the infection. Specific expression of MMP/TIMP was correlated with the type of immune response present, resolution of Cne infection and the resulting lung pathology.

Keywords C. neoformans, granuloma, MMP-2, TIMP-2

Introduction

Cryptococcus neoformans (Cne) is an opportunistic fungal pathogen that affects patients with defects in cell-mediated immunity [1,2]. T-Cell-mediated activation of macrophages is the major mechanism of host defense leading to granuloma formation that serves to limit organ damage associated with the immune response [3–5]. Murine models of pulmonary Cne infection have revealed that the genetic profile influences the type of immune response elicited [6]. C.B-17 mice clear pulmonary Cne infection through the expression of a type 1 immune response, whereas C57BL/6 mice clear Cne less efficiently and display a type 2 immune response [7,8].

Matrix metalloproteinases (MMPs) are a family of zinc-containing enzymes that are secreted in a latent form and that require activation for full proteolytic activity. MMPs have broad substrate specificity, degrading extracellular matrix (ECM) proteoglycans, laminin, fibronectin, gelatin and the globular portion of basement membrane collagens, including type IV collagen, which is a major component of basement membranes [9]. MMPs function during the immune response, supporting the migration of activated T cells as well as macrophages via the dissolution of focal contacts between receptors, such as L-selectin, and ECM components [10,11]. Macrophages secrete a variety of the proteases that contribute to degradation and remodeling of connective

Correspondence: A. A. Izzo, Department of Microbiology, Midwestern University, 555 31st Street, Downers Grove, IL 60515, USA. Tel.: +1 630 515 6173; Fax: +1 630 515 7245; E-mail: aizzox@midwestern.edu
tissues, whereas T cells secrete lower levels of gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which primarily facilitate their migration [10].

MMP expression is regulated at various levels: transcriptional, pro-enzyme activation and suppression by endogenous inhibitors. Activity is in part, regulated by tissue inhibitors of metalloproteinases (TIMPs), which bind MMPs to regulate their activity. TIMP-1 and TIMP-2 are secreted by many cell types in culture, and are found in body fluids and tissue extracts. They have been detected in emphysematous lungs and are believed to play a role in the resulting pathology [12]. TIMP/MMP complexes are minimally restrictive, reversible and usually occur in equimolar concentrations, requiring the presence of a catalytic and hemopexin domain in the protease to achieve high-affinity binding [9]. TIMP-1 primarily inhibits the activities of MMP-1, -3 and -9, whereas TIMP-2 inhibits MMP-2 through binding in a 2:1 ratio, abolishing the protease activity [13]. TIMP-2 has been shown to bind and stabilize MMP-2 by preventing autolytic degradation as well as to participate in its activation [14].

Resolution of infection by pulmonary pathogens without significant destruction of the lung parenchyma involves a balance of host immune cells, cytokines, MMPs and TIMPs. Excessive proteolysis of MMPs results in the pathology associated with lung disease. Mycobacterium tuberculosis infection has been shown to result in elevated levels of MMP-1 and -9 in lung lavage cells [15]. Phospholipase C, a virulence factor of several pathogenic bacteria, directly increases expression of MMP-3, -9 and -2 [16]. MMP-7 has also been shown in vitro to increase in human promonocytes and monocytes following lipopolysaccharide (LPS) stimulation [17].

We have used the difference in expression of immunity between two mouse strains to examine the relationship between the type of immune response expressed to pulmonary Cne infection and MMPs. In the current study, it is hypothesized that the differences in the type of immune response, type 1 vs. type 2, to pulmonary Cne infection will influence MMP production, resulting in differences in lung pathology. Results presented here show that in C.B-17 mice pulmonary MMP-2 expression is upregulated during the type 1 immune response to Cne infection. These mice have well-formed pulmonary granulomatous lesions with little tissue destruction. C57BL/6 mice that mount a type 2 immune response fail to upregulate significant levels of MMP-2 and have poorly formed cellular aggregates, but greater lung tissue destruction. These results indicate that MMP-2 may be important in granuloma formation, whereas TIMP-2 functions in maintaining granuloma integrity, preventing excessive tissue damage. The characterization of the proteolytic cascade in response to specific cell types and cytokines involved in an immune response may lead to potential targets for therapeutic intervention in destructive immune responses.

Materials and methods

Mice

C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) and C.B-17 mice (Taconic, Germantown, NY) were maintained in a filtered-air environment and given sterile food and water ad libitum. Mice were infected at 8–10 weeks of age. All procedures involving mice were carried out under aseptic conditions.

Organism

Cne strain 52D (ATCC 24067, American Type Culture Collections, Manassas, VA) was grown for 36–40 h at room temperature to early stationary phase in Sabouraud dextrose broth (1% neopeptone, 2% dextrose; Difco Laboratories, Detroit, MI).

Intratracheal infection

Mice were infected according to the protocol described previously [7,8]. Briefly, mice were anesthetized, the trachea exposed and 10⁵ Cne ml⁻¹ in a 50-μl volume was injected into the lung. To determine colony-forming units (cfu) in the lungs of infected mice, organs were homogenized and serial 10-fold dilutions were plated on Sabouraud dextrose agar (Difco, Detroit, MI).

Preparation of single cell suspensions from Cne-infected lungs

Lung cells from mice were isolated as described previously [7,8]. Total viable cell numbers were determined using Trypan Blue. Diff-Quik Stain Set (Dade Behring Inc. Newark, DE) was used to determine cell types from cytopsin preparations. Stained preparations were analyzed in a blinded fashion and two individuals performed differential cell counts.

Histology

Lungs from infected mice were inflated with formalin (Sigma Chemical Co. St. Louis, MO), excised and stored in formalin until embedded in paraffin. Lung sections were stained with hematoxylin and eosin (Fisher Scientific, Pittsburgh, PA). Photomicrographs were taken with a Zeiss microscope (Zeiss Inc, Thornwood NY).
Semi-quantitative RT-PCR

Whole lungs were removed from mice at various time points prior to and during Cne infection, homogenized in Trizol (Gibco, Gaithersburg, MD) and subjected to RNA extraction using the manufacturer’s protocol. To ensure the quantity and integrity of the RNA 5 µg was electrophoresed on a formaldehyde gel on which the 18S and 28S ribosomal bands were visualized by ethidium bromide staining. Superscript II (Gibco) was used for cDNA synthesis using 2 µg of RNA and oligo(dT) primers. The reverse transcriptase (RT) reactions were brought to 100 µl, 5 µl of which was used in standard magnesium-containing PCR reactions to identify MMP-2 (Forward: 5’-CTATTCTGTCAGCAGTTGG, Reverse: 5’-CAGAATTTGTTTCTTCAACTT), TIMP-2 (Forward: 5’-GACCCAGTCCATCCAGAGGC, Reverse: 5’-GAGATCAAGCGAGATAAAGATG) and 18S rRNA (Forward: 5’-GAGCTCAGGGTTGGTATTG, Reverse: 5’-TACCTGGTTGATCCTGCGAG). The resulting PCR products were electrophoresed on 1.2% agarose gels and photographed using a Kodak Polaroid (Cambridge, MA) camera. The black and white photographs were scanned using a UMAX Astra 1200S (Dallas, TX) and quantified using NIH Image Analysis (Bethesda, MD) software. The density of each band was normalized to that of 18S rRNA for each group of samples for semi-quantitation.

Statistical analysis

Mann-Whitney test was used to analyze lung colony-forming unit (cfu) values.

Results

Pulmonary leukocyte recruitment and granuloma formation

C.B-17 and C57BL/6 mice differ in their ability to resolve pulmonary Cne infection and in the cytokines produced by infiltrating leukocytes during infection [7,8]. Expression of adaptive immunity in both strains is generated from about day 7 of infection [3-5]. Mice were infected with Cne and cfu were then determined in their lungs. Table 1 shows lung cfu in C.B-17 and C57BL/6 mice at days 14 and 28 of infection and agrees with previously published data [8]. Differences in the types of cells recruited into the lungs of Cne infected C.B-17 and C57BL/6 mice at days 14 and 28 of infection also confirms data reported by others (Fig. 1) [8]. At day 14 of infection recruitment of macrophages into infected lungs was greater in C.B-17 mice than in C57BL/6 mice, but by day 28 of infection the number of macrophages was similar in both strains. A difference between the mouse strains was observed in the recruitment of eosinophils into the lungs of C57BL/6 mice at day 28 of infection, accompanied by a type 2 immune response [8]. The absence of eosinophils in the lungs of C.B-17 mice correlated with expression of a type 1 immune response and was associated with recruitment and activation of macrophages. By day 28 of infection C.B-17 mice had resolved infection, whereas C57BL/6 mice had a significantly greater Cne burden (Table 1).

Histologic examination of the lungs at day 14 of infection showed recruitment of neutrophils and macrophages into sites surrounding Cne in both C.B-17 and C57BL/6 mice (data not shown). Accumulations of lymphocytes were also evident in these areas, suggesting the development of immunity to infection. At day 28 of infection when C.B-17 mice were resolving infection, well-formed pulmonary granulomas that contained Cne were evident (Fig. 2a) as well as large areas of the lung with normal morphology. In contrast, C57BL/6 mice did not have well-formed granulomas and their lungs contained scattered accumulations of macrophages (Fig. 2b) as well as eosinophilic infiltrates dispersed throughout the lungs.

MMP and TIMP expression following pulmonary Cne infection

RNA from the lungs of C.B-17 and C57BL/6 mice were examined for the expression of MMP-2 and TIMP-2 at
days 0, 14 and 28 of infection by RT-PCR. Figure 3(a, b) shows the semi-quantitative determination of mRNA expression for MMP-2 and TIMP-2 in the lungs of C.B-17 and C57BL/6 mice at days 0, 14 and 28 of infection. MMP-2 mRNA in the lungs of C.B-17 mice was detected at days 14 and 28 of infection, the time at which granulomatous lesions formed within the lungs of these mice. In the lungs of C57BL/6 mice, which did not form well-defined granulomas, MMP-2 mRNA was below detectable levels until day 28 of infection, and then was present at much lower levels than that seen in C.B-17 mice. TIMP-2 mRNA expression was consistently elevated in the lungs of both mouse strains throughout infection. At day 14 of infection in C.B-17 mice, at the height of the immune response, the relative levels of MMP-2 and TIMP-2 had reversed compared with those seen in naive lungs, with increased expression of MMP-2 mRNA and decreased TIMP-2 mRNA. At day 28 of infection, the ratio of the two RNA types was closer to one and coincided with the formation of pulmonary granulomatous lesions in these mice.

Taken together, the data suggest that the expression of MMP-2 early during Cne infection was required for

<table>
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<tr>
<th>Mouse strain</th>
<th>Mean log_{10} cfu (SD)</th>
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<tr>
<td></td>
<td>Day 14</td>
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<tr>
<td>C.B-17</td>
<td>6.62 (0.02)</td>
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<tr>
<td>C57BL/6</td>
<td>7.05 (0.21)</td>
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Table 1  Colony forming units (cfu) of Cne in the Lungs of C.B-17 and C57BL/6 mice. Mice were infected with ≈ 250 cfu of Cne strain 52D via intratracheal inoculation. At days 14 and 28 of infection lung cfu were determined by plated lung homogenates on Sabouraud dextrose agar. Values represent the mean log_{10} cfu (SD) from five mice per time point.

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Fig. 2 Photomicrographs of lungs of C.B-17 (a) and C57BL/6 (b) mice at day 28 of infection. Serial sections from infected lungs from each strain were stained with hematoxylin and eosin and examined to identify granulomatous lesions. Magnification is ×100.

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promoting granuloma formation in the lungs of C.B-17 mice during infection. Altering the ratio of MMP-2 to TIMP-2 may also play a role in downregulating the activity of MMP-2 once granulomas have formed. This may be important in maintaining the architecture of the granulomas.

Discussion

An established model of pulmonary Cne infection [38] was used to examine the function of matrix metalloproteinases in the lung during infection. In this model, outcome of infection depended on the type of immune response mounted; C.B-17 mice developed a type 1 immune response and resolved infection, whereas C57BL/6 mice developed a type 2 response resolved infection less efficiently. In addition, C.B-17 mice formed pulmonary granulomatous lesions that contained Cne and the immune response was localized to these areas, leaving the large areas of normal lung (Fig. 3). In contrast, infection in C57BL/6 mice produced lungs full of loose aggregates of macrophages, lymphocytes and eosinophils with large numbers of Cne present that had no distinct organization. We hypothesized that granuloma formation was affected by the expression of MMPs by recruited leukocytes that enabled tissue remodeling to form organized structures. The elevated expression of MMP-2 mRNA at days 14 and 28 of infection in the lungs of C.B-17 mice (Fig. 3) suggests that MMP-2 may contribute to tissue remodeling associated with granulomatous lesion formation.

Pro-MMP-2 is activated through a tri-molecular complex that also involves MT1-MMP (MMP-14) and TIMP-2 (14). Increased concentrations of TIMP-2 inhibit the process by binding to MMP-14. Our study examined the relationship between MMP-2 and TIMP-2 during pulmonary granuloma formation in response to Cne infection. In the absence of granulomatous lesions as observed in C57BL/6 mice, MMP-2 was not detected at the mRNA level until later on during infection, and then only at very low levels compared with C.B-17 mice. C.B-17 mice, which formed pulmonary granulomas, had elevated levels of MMP-2 mRNA at times when immunity to Cne was greatest. Our findings do not preclude the possibility that other MMPs and TIMPs may also be involved in the process, and further investigations are planned to examine the relationship between MMP-2 expression and granulomatous lesion formation. Studies involving pulmonary granuloma formation during M. tuberculosis infection have shown MMP-2 up-regulation within granulomatous lesions (A.A. Izzo, unpublished data).

Macrophages secrete a variety of MMPs when stimulated [18]. Rat alveolar macrophages markedly upregulated MMP-9 and MMP-12 upon stimulation [19,20]. Although early during the infection alveolar macrophages do play a significant role in limiting infection and elaborating MMPs, macrophages that are recruited into chronic inflammatory sites may be induced to secrete a different profile of MMPs and TIMPs. Human monocytes

Fig. 3 (a) Amplified RT-PCR products were analyzed by electrophoresis through 1.2% agarose gel containing ethidium bromide (b) Semi-quantitative RT-PCR analysis of MMP-2 and TIMP-2 in the lungs of Cne infected C.B-17 (□) and C57BL/6 (■) mice. Amplified products were scanned and the expression of each product was then standardized to that of the housekeeping gene, 18S rRNA to obtain the Relative Pixels value. The mRNA from whole lung preparations from 3–4 mice per time point was used for analysis of MMPs and TIMPs.
and macrophage cell lines infected with *M. tuberculosis* upregulated MMP-1 and MMP-9 expression [15].

There have been no reports of Cne-produced cyto-
toxins that directly damage host tissue, although Cne was shown to possess a metalloproteinase-like peptidase that cleaved collagen [21]. MMP-like molecules have been reported in several bacteria and other fungi and these enzymes may provide a mechanism for organisms to disseminate from organ to organ or may function to activate the host’s MMPs [22]. During situations in which the immune response cannot efficiently control infection, as observed in C57BL/6 mice, the presence of high numbers of Cne for extended periods may directly mediate lung damage. In contrast, if the infection can be controlled as observed in C.B-17, mice then concentrations of these MMP-like enzymes may not have a marked effect on the lung architecture.

The current studies show that granuloma formation in response to pulmonary Cne infection is associated with the expression of MMP-2 in the lung. Of particular interest in granuloma formation is the role of tumor necrosis factor-α (TNF-α), which is known to regulate expression of MMPs in many systems [23] as well as to function in maintaining granuloma architecture [24]. TNF-α is an essential component in the expression of immunity to Cne and may also have a function in granuloma formation [25]. Further studies are also planned to investigate the function of MMP-2 during granuloma formation by using inhibitors of MMP to determine its role in tissue remodeling during this process.

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