Active Matrix Metalloprotease-9 Is Associated with the Collagen Capsule Surrounding the Madurella mycetomatis Grain in Mycetoma

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Abstract

Madurella mycetomatis is the main causative organism of eumycetoma, a persistent, progressive granulomatous infection. After subcutaneous inoculation M. mycetomatis organizes itself in grains inside a granuloma with excessive collagen accumulation surrounding it. This could be contributing to treatment failure towards currently used antifungal agents. Due to their pivotal role in tissue remodelling, matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) play a pivotal role. MMPs are zymogens, proteolytic activation is prerequisite to become completely active. Although it seems paradoxical, inhibition of MMP activity with doxycycline could prevent collagen accumulation in mycetoma, which in turn might make the fungus more accessible to antifungal agents.

Introduction

Madurella mycetomatis is the most prevalent causative organism of eumycetoma, a persistent, progressive granulomatous infection involving subcutaneous tissues and bones [1]. Mycetoma lesions are characterized by subcutaneous masses, sinuses and fungal grains, which commonly progress under inappropriate treatment resulting in deformation and disabilities of infected body parts [1]. To treat eumycetoma, a combination of surgery and treatment with antifungal agents is required [2]. Treatment with currently available antifungal agents, ketoconazole and itraconazole, only facilitates surgical removal of mycetoma lesions as they currently used antifungal agents, ketoconazole and itraconazole, with antifungal agents is required [2]. Treatment with the active form of MMP-9 in mycetoma patients suggest that MMP-9 is activated or synthesized by inflammatory cells upon infection. Inhibiting MMP-9 activity with doxycycline could prevent collagen accumulation in mycetoma, in which in turn might make the fungus more accessible to antifungal agents.

Macrophage metalloestase (MMP-12), and membrane-type MMP (MMP-14 to MMP-25) [6]. MMP-2 and MMP-9 have the ability to degrade a variety of ECM constituents (e.g. gelatin, elastin, and various types of collagen) [6,7]. Since both MMP-2 and MMP-9 are zymogens, proteolytic activation is prerequisite to become completely active [7]. Although it seems paradoxical, inhibition of MMP by a synthetic inhibitor decreased collagen accumulation in peritoneal sclerosis rats and bleomycin-induced pulmonary fibrotic rats [6,9]. In addition, accumulation of collagen is correlated with MMP-2 or MMP-9 in several pathological conditions such as atherosclerosis [10], cardiac fibrosis in diabetic patients [11], and granulomatous fibrosis of rats with Angiostrongylus cantonensis infection [12], suggesting that collagen deposition can be promoted by gelatinases. Although the exact mechanism(s) explaining these observations have to be clarified, it is hypothesized that MMPs induce de novo ECM accumulation through its digestion of ECM constituents. Another explanation might be that MMPs provoke collagen accumulation via another pathway than ECM digestion.

Neither TIMP-1 nor MMP-2 and MMP-9 have been described to be involved in mycetoma pathogenesis. In this study, it is determined if MMP-2 and MMP-9 were expressed locally in the...
obtained show that MMP-9 is associated with mycetoma. In addition, polymorphisms in promoter regions of MMP-2, MMP-9 and tissue inhibitors of metalloproteinases (TIMPs), these enzymes could play a role in the formation of the collagen capsule surrounding the fungal grain. Indeed, we demonstrated that MMPs were found surrounding the mycetoma grain and that measurable levels of both MMPs were found in serum of both mycetoma patients and healthy controls. Only in mycetoma patients the active form MMP-9 was found. The presence of active MMP-9 in the serum of mycetoma-patients was not the result of lower levels TIMP-1 but more likely from differences in allele frequencies in the TIMP-1 gene. In conclusion, our results showed an increased MMP-9 activity in mycetoma patients. We hypothesize that inhibition of MMP-9 activity by doxycycline will result in breakdown of the collagen capsule surrounding the grain, which in turn will make the entrance of antifungal drugs into the grain easier.

Materials and Methods

Subjects

Genomic DNA of 125 M. mycetomatis infected patients from Sudan (72.8% male; 27.2% female) and 103 healthy endemic controls were used to determine levels of MMP-2, MMP-9, and TIMP-1. The patients’ demographic characteristics were recorded and that included gender, duration of disease, lesion size and site of infection.

Sirius red staining

To localize collagen fibres, tissue sections of 8 M. mycetomatis infected subjects were stained with Sirius red staining and subsequently photographed.

Immunohistochemical staining

Immunohistochemical staining was used to assess whether MMP-2 and MMP-9 are expressed around the fungal grain. Deparaffinised tissue sections of the same 8 M. mycetomatis infected subjects used for the Sirius red staining were treated with 0.3% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity. To inhibit aspecific binding of primary antibodies, specimens were incubated for 1 hour with 2% normal goat serum in PBST (0.05% Tween20 (Sigma, Zwijndrecht, The Netherlands) in PBS. Tissue sections were incubated overnight at 4°C with primary antibodies against MMP-2 (40 μg/ml; IM33 Calbiochem) and MMP-9 (40 μg/ml; IM09L, Calbiochem). After 1 hour incubation with goat anti-mouse IgG HRP-conjugated antibody (1:200; Dako, Heverlee, Belgium) at RT, immunoreactivity was visualized using 3-aminio-9-ethyl-carbazole (AEC; Sigma, Zwijndrecht, The Netherlands). Mayer’s hematoxylin (Sigma, Zwijndrecht, The Netherlands) was used for counterstaining. As a negative control, tissues were stained without the primary antibodies being used.

ELISA

Absolute serum levels of MMP-2 and MMP-9 in serum of M. mycetomatis infected patients (n = 36) and healthy controls (n = 36) were determined utilizing Human MMP-2 and Human MMP-9 enzyme-linked immunosorbent assay (ELISA) kits (cat#: RAB0365, Sigma–Aldrich, Zwijndrecht, The Netherlands; and cat#: KHC3061, Invitrogen, Breda, The Netherlands). Human TIMP-1 ELISA kit (Cat#: OK-0163, Assay Biotechnology, Breda, The Netherlands) was used to assess the serum level of TIMP-1 in both study populations (n = 44 for both populations). Experiments were conducted according to the manufactures instructions.

Gelatin zymography

Gelatinolytic activity in sera of M. mycetomatis infected patients and healthy endemic controls were determined by gelatin zymography. One μl serum was electrophoresed under non-reducing conditions on a 10% SDS-polyacrylamine gel copolymerized with 1 mg/ml gelatin (Fluka Analytical, Zwijndrecht, The Netherlands). As a positive control 0.4 ng activated proenzyme MMP-2 and 0.1 ng activated proenzyme MMP-9 (Enzo Life Sciences, Antwerp, Belgium) were used. After incubating the gel four times in 2.5% Triton X-100 (v/v) (Sigma, Zwijndrecht, The Netherlands) for 15 minutes, the gel was incubated in developing buffer (50 mM Tris (pH 7.5; Sigma, Zwijndrecht, The Netherlands), 200 mM NaCl (Merck, Amsterdam, The Netherlands), 5 mM CaCl2 (Merck, Amsterdam, The Netherlands) and 0.02% Brij35 (Calbiochem, San Diego, USA) for 65 hours at 37°C. The gel was stained with 50% methanol (Fisher Scientific, Landsmeer, The Netherlands), 20% acetic acid (J.T. Baker, Deventer, The Netherlands), and 0.125% Coomassie Brilliant Blue R-250 (Sigma, Zwijndrecht, The Netherlands) and destained with destaining solution (30% methanol and 1% formic acid (J.T. Baker, Deventer, The Netherlands) until transparent lysis bands were visible.

Genotyping of MMP-2, -9, and TIMP-1 polymorphisms

Functional SNPs in promoter regions of MMP-2, MMP-9, and TIMP-1 were determined utilizing genomic DNA of 125 M. mycetomatis infected patients and 103 healthy controls. To determine MMP-2 (−1306 C/T) genotype, DNA was amplified as described before [16,17] and amplified using primers 5’-CTTCTCTGGTGCCTTACTA-GT-3’ and 5’-CTGAGACCTGAAGGCTGAAGAGAGCT-3’. The PCR reaction consisted of 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C and 30 s elongation at 72°C. The genotype of the resulting amplicon was determined by restriction fragment length polymorphism (PCR-RFLP) with Sphi. To determine the MMP-9 (−1562 C/T) genotype, DNA was amplified using primers 5’-GGCTGGGAGATGAGGCC-3’ and 5’-CCTTGGAAGGGATAGGCC-3’. The PCR reaction was similar to the one described for the MMP-2 (−1306 C/T) polymorphism, only the annealing temperature was changed to 65°C. The genotype of the resulting amplicon was determined by restriction

Author Summary

Eumycetoma, mainly caused by the fungus Madurella mycetomatis, is a chronic infection which, without treatment, results in deformation of the infected body part. Inside the body, the fungus organises itself in grains which are surrounded by collagen. This collagen could act as a natural barrier for antifungal agents. Since collagen modulation is regulated by matrix metalloproteinase-2 (MMP-2), MMP-9 and tissue inhibitors of metalloproteinases (TIMPs), these enzymes could play a role in the formation of the collagen capsule surrounding the fungal grain. Indeed, we demonstrated that MMPs were found surrounding the mycetoma grain and that measurable levels of both MMPs were found in serum of both mycetoma patients and healthy controls. Only in mycetoma patients the active form MMP-9 was found. We hypothesize that inhibition of MMP-9 activity by doxycycline will result in breakdown of the collagen capsule surrounding the grain, which in turn will make the entrance of antifungal drugs into the grain easier.
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Statistical analysis
Deviation from Hardy-Weinberg equilibrium for each polymorphism was calculated by the Pearson’s χ² test. Differences in categorical variables and continuous variables between M. mycetomatis infected patients and reference group were tested with Fisher’s exact or Mann-Whitney test respectively. Statistical comparisons were carried out using GraphPad Prism 5.0 or GraphPad InStat 3.0 (GraphPad Software, San Diego California USA). P<0.05 was considered to be statistically significant.

Ethics statement
Written informed consent was obtained from all participants and ethical clearance was obtained from Soba University Hospital Ethical Committee, Khartoum, Sudan.

Results
Excessive collagen deposition surrounds the fungal grain
Collagen accumulation around the fungal grain was assessed by staining specimens of M. mycetomatis infected subjects by Sirius red. A representative photomicrograph of a Sirius red stained tissue section shows that the fungal grain is encapsulated with collagen deposition (Figure 1b). After this first collagen deposition ring, often a denser collagen capsule is seen at some distance of the grain. In that capsule typical collagen fibres are noted.

MMP-2 and MMP-9 are expressed by immune-cells surrounding the fungal grain
In order to determine if the gelatinases MMP-2 and MMP-9 play a role in the encapsulation of the mycetoma grain, the presence of these two MMPs was demonstrated by immunohistochemical staining of tissue sections of patients infected with M. mycetomatis (Figures 1c and 1d). As is seen in figure 1c and 1d, both MMP-2 and MMP-9 were detectable as red cytoplasmatic staining in cells, mainly in zone 2 surrounding the grain. In the neutrophil zone (zone 1), little expression of either metalloproteases was noted, although in some patients also this zone showed expression of MMP-2 and MMP-9. Strikingly expression was mainly found in areas where little collagen deposition was seen. If there was heavy collagen deposition hardly any MMP-2 and MMP-9 expression was noted (figure 1). In slides where primary antibodies were omitted, coloration was absent (not shown).

Active MMP-9 in serum is detectable in mycetoma patients but not in healthy endemic controls
In order to determine if the MMP-2 and MMP-9 expression was also found in serum, ELISAs were performed to determine the concentrations of MMP-2 and MMP-9 in sera of mycetoma patients and healthy controls. It appeared that MMP-2 was hardly detected in sera of either patients or controls (Figure 2A, MMP-2 median 0 ng/ml for both groups). There were no differences between the patients and the healthy controls (Mann-Whitney, p = 0.42). Also, similar concentrations of MMP-9 were found in sera of both M. mycetomatis infected patients and controls (Figure 2B, median concentration 451.6 ng/ml versus 461.2 ng/ml, respectively; Mann-Whitney, p = 0.57). The drawback by measuring MMP-2 and MMP-9 concentrations by ELISA is that it is not possible to distinguish between inactive and active MMP-2 and MMP-9. In order to distinguish between active and inactive gelatinase in sera of M. mycetomatis infected patients and healthy endemic controls, gelatin zymography was used. Characteristic gelatinolytic patterns due to the presence of pro-active and active forms of MMP-2 and MMP-9 in sera of M. mycetomatis infected patients and healthy controls are depicted in Figure 3. Active MMP-9 of 94 kDa was found in sera of 36% of M. mycetomatis infected subjects, whereas this active form was not present in sera of the control population (Fisher Exact, p<0.0001). No correlation was found between the presence of active MMP-9 and lesion size or disease duration (data not shown). Active MMP-2 (62 kDa) was absent in both groups. The pro-active forms of MMP-2 and MMP-9, 72 and 92 kDa respectively, were present in sera of all M. mycetomatis infected patients and healthy controls. Since mycetoma patients had more often active MMP-9 in their sera, while the total amount of MMP-9 (both active and inactive) did not differ, it was investigated if TIMP-1 levels differed between patients and healthy endemic controls by ELISA. This was done since TIMP-1 is known to block protease activity of both MMP-2 and MMP-9. It appeared that TIMP-1 serum levels of both groups did not significantly differ (Figure 2C, median 195.8 ng/ml for M. mycetomatis infected patients vs. 170.0 ng/ml for controls; Mann-Whitney, p = 0.99). Ratios of MMP-9 to TIMP-1 were comparable and did not reach statistical significance (Figure 2D; Mann-Whitney, p = 0.59).

The T-allele of the TIMP-1 (+372 C/T) polymorphism is associated with mycetoma in males
In order to determine if the difference in active MMP-9 levels was the result of genotypic differences between patients and healthy controls, we determined whether allele frequencies in functional polymorphisms in MMP-2, MMP-9 and TIMP-1 differed between M. mycetomatis infected patients and healthy controls by SNP analyses. All studied genotypes did not show deviation from Hardy-Weinberg equilibrium (p>0.05). Allele frequencies of MMP-2, MMP-9, and TIMP-1 polymorphisms were compared between M. mycetomatis infected patients and healthy controls (Table 1). Since the TIMP-1 gene is X-chromosome located, genotype analyses were stratified according to gender.

The allele distributions for MMP-2 (−1306 C/T) and MMP-9 (−1562 C/T) polymorphisms did not significantly differ between M. mycetomatis infected patients and controls and (p = 0.39 and p = 1.00 respectively). The T allele frequency in TIMP-1 (+372 C/T) polymorphism was significantly higher in male M. mycetomatis infected patients compared to the male reference group (46% versus 26%) (p = 0.0004). In female M. mycetomatis infected patients the allelic distribution in TIMP-1(+372 C/T) polymorphism did not significantly differ with female control subjects (p = 0.53).

Discussion
Eradication of M. mycetomatis mycetoma remains challenging as in vivo treatment failure towards currently used antifungal agents is frequently observed. It has been reported that collagen accumulation contributes to limited penetration of chemotherapeutic agents into the granuloma [18], suggesting that a dense collagen network might influence drug accessibility. Therefore, diminished response upon antifungal treatment might be partly caused by excessive collagen accumulation in the mycetoma lesion. Unravelling the mechanism behind observed changes in tissue architecture around the fungal grain could direct to novel therapeutic options. In this study we investigated suitable candidates, MMP-2, MMP-9, and TIMP-1, as they are participants in ECM remodeling.
Both MMP-2 and MMP-9 were found to be expressed in the mycetoma lesion, and both were highly expressed locally surrounding the fungal grain. Constitutive expression of MMP-2 takes place in various cell types and is barely induced under pro-inflammatory conditions [19]. Furthermore, one of the characteristics of mycetoma is that during grain formation high amounts of neutrophils are recruited to the site of *M. mycetomatis* infection [17]. MMP-9 is constitutively expressed and stored in high quantities in granules of neutrophils, and several chemotactic chemokines and cytokines are able to induce degranulation of MMP-9 containing granules [20,21]. Furthermore, inflammatory stimuli are able to upregulate MMP-9 expression in a wide range of inflammatory cell types, such as lymphocytes, monocytes, and neutrophils [19,22].

Absolute MMP-2, MMP-9, and TIMP-1 serum levels were comparable between *M. mycetomatis* infected patients and healthy controls and did not reach statistical significance. However, since the ELISA measured both the pro-active and the active forms of MMP-2 and MMP-9, these observations have only a limited value. Therefore, MMP-2 and MMP-9 activity was tested by gelatin zymography. Despite comparable absolute serum levels in both groups, MMP-9 activity was significantly higher in the *M. mycetomatis* mycetoma population. A higher MMP-9 activation could be the result of a higher MMP-9 expression or a lower TIMP-1 expression. TIMP-1 inhibits MMP-9 activity by forming a 1:1 stoichiometric non-covalent complex [6]. Disruption of MMP-9:TIMP-1 complexes result in release and activation of MMP-9. Several other participants in MMP-9 activation have been described, including protease-based activators (e.g. trypsin [23] and neutrophil-derived elastase [24]) and other MMPs [21]. Although we only found activated MMP-9 found in mycetoma patients, there was still a large proportion of the patients in which we did not find the activated form. Similar findings were reported for patients with severe sepsis [25]. Only in 10 out of 20 patients with severe sepsis on the intensive care unit, activated MMP-9 was found [25]. Again no correlation with disease severity was noted.

Figure 1. Collagen deposition and MMP-2 and MMP-9 expression around the *M. mycetomatis* grain. In this figure a representative picture of the *M. mycetomatis* grain insight the subcutaneous tissue from one particular patient is shown. In panel A, a HE staining is performed. The grain (G) and two different zones surrounding the grain are clearly visible (1,2). Zone 1, representing the neutrophil zone is relatively small around this typical grain. In zone 2, histocytes, capillaries, lymphocytes, plasma cells, fibroblasts and some macrophages are seen. In panel B, a Sirius red staining of the same area is shown. As is seen on this slide, collagen (coloured red) is mainly seen within zone 2. In panels C and D, MMP-2 and MMP-9 are detected by immunohistochemical staining, respectively. MMP-2 and MMP-9 positive cells are stained red, as a counterstaining hematoxylin is used. Relatively little MMP-2 positive cells were noted in the rim of zone 2, outside the layer where the collagen deposition was seen in panel B. MMP-9 staining was more heavily. Some cells in zone 2 stained positive for MMP-9, but more positive cells were seen at the rim of this zone (zone 3). No MMP-2 expression was noted in zone 1 surrounding the grain.

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Why we measured in one patient active MMP-9 and the other not is not clear. Several reasons could be attributing. First of all, we only took one time-point and these time-points differ for each patient. MMP-9 expression could be dependent on the disease stadium, although we did not find a correlation with the disease duration or the size of the lesion, other factors might be responsible such as if the patient had at the time of sampling discharging sinuses or not. Furthermore we did not record if the patient had other infections. Furthermore, it is also plausible that co-infections could play are role since they are frequently reported in mycetoma [26] and a correlation between mycetoma and schistomiasis was also recently reported [27]. Differences in MMP-9 expression between the M. mycetomatis infected patients individually and between patients and healthy endemic controls as a group could also be caused by genetic differences. We therefore genotyped functional polymorphisms in the promoter regions of MMP-2 (−1306 C/T), MMP-9 (−1572 C/T), and TIMP-1 (+372). While no significant difference in allele distributions in the MMP-9 (−1572 C/T) polymorphism was found, other SNPs in the promoter region of MMP-9 or MMP-9 itself were not investigated and could contribute to increased MMP-9 activation in M. mycetomatis infected patients. In contrast, a genetic difference between both groups was found for TIMP-1 (+372 SNP). The T allele frequency in TIMP-1 SNP in male M. mycetomatis infected patients was significantly higher compared to healthy controls. In man, T allele associated transcriptional activity of TIMP-1 is lower than C allele associated transcriptional activity [15], suggesting that TIMP-1 production and thereby MMP inhibition in these subjects is reduced. This finding might explain previously reported male predominance in mycetoma [1]. Due to lower T allele associated transcriptional activity of TIMP-1, we expected reduced TIMP-1 serum levels in M. mycetomatis infected subjects, but this was not the case. However, since mycetoma is a localized infection, a localized reduction of TIMP-1 could result in higher

Figure 2. Concentrations of MMP-2 (A), MMP-9 (B), TIMP-1 (C), and MMP-9:TIMP-1 ratios (D) in sera of M. mycetomatis infected subjects and healthy endemic controls. Data is presented as median. Each dot represents the serum concentration or ratio of each (inhibitor of) metalloproteinases of one patient or healthy control. Significance was determined by Mann-Whitney test.
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Figure 3. MMP activity in sera of M. mycetomatis infected patients and healthy endemic controls as demonstrated by gelatine zymography. One µl of serum of each subject was analyzed for MMP activity. Representative gelatine zymograms of 12 M. mycetomatis infected subjects (A) and 10 healthy endemic controls (B) are shown. The pro-active and active forms of MMP-2 and MMP-9 are marked with arrows. MMP-2: Activated proenzyme MMP-2; MMP-9: Activated proenzyme MMP-9.
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MMP-9 levels in the lesion, which, in its turn, could be found in serum.

In this study we showed that collagen is indeed encapsulating the grain and MMP-9 is the collagenase activated during *M. mycetomatis* infection. The question remains what the exact function of grain encapsulation is. Is this encapsulation beneficial to the host, by keeping the *M. mycetomatis* infection localized and preventing the spread of infection? Or prevents the collagen capsule surrounding the fungal grain the penetration of drugs into the grain? If the latter would be the case, one could consider adding the antimicrobial agent doxycycline to the currently used therapeutic strategy. Doxycycline is a potent MMP inhibitor which is able to reduce MMP-2 and MMP-9 mRNA expression and MMP-2 production in *in vitro* and thereby attenuates collagen accumulation in pulmonary fibrosis [28]. By attenuating the collagen deposition around the grain, ketoconazole and itraconazole might be able to better penetrate to the fungus.

In summary, the results obtained in the present study show increased MMP-9 activity during *M. mycetomatis* infection, suggesting that MMP-9 is associated with *M. mycetomatis* mycetoma.

**Author Contributions**

Conceived and designed the experiments: KG WK AHF WWJvdS. Performed the experiments: KG WK WWJvdS. Analyzed the data: KG WK WWJvdS. Contributed reagents/materials/analysis tools: KG WK AHF WWJvdS. Wrote the paper: KG WK AHF WWJvdS.

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