Original Article

Expression of Matrix Metalloproteinases (MMP-3 and MMP-9) in Skin Lesions of Leprosy Patients

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Background. Leprosy is a chronic granulomatous disease, caused by *Mycobacterium leprae (M. leprae)*, principally affecting peripheral nerves and skin. To date, the role of matrix metalloproteinases (MMPs) in different types of leprosy has not been studied, except for their role in neuropathy. **Objective.** to investigate the changes in expression and distribution of MMP-3 and MMP-9 in biopsies taken from cutaneous lesions of leprosy patients. **Patients and Methods.** Using immunohistochemistry, 24 leprosy lesional skin biopsies compared with 20 matched healthy controls biopsies were assessed for expression of MMP3 and MMP9. Patients were divided into 4 groups; group I tuberculoid (TT) and borderline tuberculoid (BT), group II borderline borderline leprosy (BB), group III borderline lepromatous (BL) and lepromatous leprosy (LL), and group IV erythema nodosum leprosum (ENL). **Results.** MMP-3 is increased in type 2 lepra reaction; ENL, in endothelial cells of blood vessels of vasculitic lesions and in extracellular matrix (ECM), and fibroblasts around infiltrating cells. MMP-9 expression was mainly observed in functioning macrophages; epithelioid cells of the paucibacillary (PB) tuberculoid pole of leprosy spectrum, and immunoreactivity was decreased towards the multibacillary (MB) lepromatous pole, characterized by impaired-functioning foamy histiocytes. **Conclusion.** MMP-3 has a possible role in vasculitis process of ENL. MMP-9 is a key marker of functioning macrophages, epithelioid granuloma formation, and potential destruction of peripheral nerves of PB leprotic patients. Natural inhibitors of MMPs do exist, and synthetic inhibitors have been developed which offer the hope of new adjuvant treatment options in leprosy. (*J Egypt Women Dermatol Soc* 2009; 6: 80-87)

Keywords. MMP-3, MMP-9, leprosy

Leprosy is a chronic granulomatous disease, caused by *Mycobacterium leprae (M. leprae)*, principally affecting peripheral nerves and skin. The pathogenesis and thus the clinical features reflect variable degree to which cell-mediated immunity (CMI) is expressed[1]. Lepromatous leprosy (LL) represents a failure of CMI specifically towards *M. leprae*, with absence of activated lymphocytes and macrophages, meaning that nerve damage is slow and gradual. In tuberculoid leprosy (TT), CMI is strongly expressed so that the infection is restricted to one or a few skin sites and peripheral nerves. Between those two polar forms lie the borderline forms of the disease, with the extent of the disease reflecting the balance between CMI and the bacillary load[2]. Borderline patients; borderline tuberculoid (BT), borderline (BB), and borderline lepromatous (BL), are immunologically unstable and at risk of developing type 1 (reversal) reactions which are delayed hypersensitivity reactions caused by increased recognition of *M. leprae* antigens in skin and nervers[3]. Type 2 reactions; erythema nodosum leprosum (ENL) are due to immune complex deposition and occur in BL and LL patients[2].

Matrix metalloproteinases (MMPs) belong to a family of zinc-dependent extracellular proteases, collectively able to degrade all components of the extracellular matrix (ECM) and share extensive substrate overlap[4]. MMPs can be classified broadly by substrate specificity into collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, -11), elastases (MMP-7 and -12) and membrane-type MMPs (MT-MMPs; MMP-14, -15, -16 and -17), which are surface anchored by either a trans-membrane domain or a glycosyl-phosphatidylinositol anchor. The catalytic domain is highly conserved and determines substrate specificity[5]. The proteolytic activity of MMPs is inhibited by specific tissue inhibitors of metalloproteinase (TIMPs), with which they form 1:1 complexes. The MMP: TIMP ratio is critical in regulating the proteolysis of connective tissues and in controlling tissue damage[6].

Although MMPs were considered initially as purely matrix-degrading enzymes, their known functions have expanded beyond ECM remodeling

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to include a plethora of biological roles in cellular migration, immunomodulation, angiogenesis and matrix remodeling[7-9]. MMPs are secreted by both inflammatory and stromal cells in response to both exogenous insults and inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β[10,11]. Additionally, cell contact-dependent signaling may drive MMP up-regulation[12,13]. On the other hand, MMP secretion is down-regulated by diverse cytokines including interferon-gamma (IFN-γ), IL-4 and IL-10[14], although the regulation of secretion is cell- and stimulus-specific[6].

In the skin, stromelysin 1 (MMP-3) is normally expressed in keratinocytes and fibroblasts, while gelatinase B (MMP-9) is produced by keratinocytes, monocytes and macrophages[15]. In the normal immune response to infection, successful eradication of infection by the host requires the influx of effector cells, killing of the pathogen, resolution of inflammation and finally remodeling of the ECM. Host-derived MMPs are necessary for the successful execution of these events, by facilitating leukocyte recruitment, cytokine and chemokine processing, defensin activation and matrix remodeling[16]. However, excess MMP activity following infection may lead to immunopathology that causes host morbidity or mortality and favors pathogen dissemination or persistence[5].

Previous studies showed that MMP-3 is essential for initiation of immune response, by enhancing leukocyte transmigration to the site of inflammation and across high endothelial venules into lymph nodes[17], where antigen will be detected[18,19]. Besides, T cell and dendritic cell migration was found to be, in part, MMP-9-dependent[20]. Nevertheless, MMP-9 can stimulate IL-10 production; a powerful inhibitor of delayed hypersensitivity reactions, by keratinocytes and/or macrophages, possibly leading to tolerance[18,21].

To date, the role of MMPs in different types of leprosy has not been studied, except for their role in neuropathy. Teles et al.[22] studied the immunoreactive profiles for MMPs (including MMP-9), and demonstrated strong reactivity of myelinated axons, infiltrating macrophages, Schwann cells, endothelial cells, and perineural cells in neuritic leprosy biopsies. This study therefore aims to investigate the changes in expression and distribution of MMP-3 and MMP-9 in biopsies taken from cutaneous lesions of leprosy patients.

PATIENTS AND METHODS

Patients. Twenty-four newly diagnosed untreated leprosy patients (11 males and 13 females), with average age of 40.4 ± 18.2 years, were included in this study. They were collected from the out-patient clinic of Dermatology Department, Ain Shams University Hospital in Cairo and Dermatology and Leprosy Hospital in Tanta City. Diagnosis of leprosy was based on the WHO definition of leprosy case[23,24]. Patients were evaluated according to clinical examination, skin smear examination and histopathological examination. Patients were further divided into 4 groups: Group I: seven patients with TT/BT, Group II: five patients with BB, Group III: six patients with BL/LL, and Group IV: six patients with BL/LL in type 2 reaction or ENL. Patients who started treatment or were on any kind of immunomodulatory or immunosuppressive therapy likely could alter the results of the study, such as systemic corticosteroids, were excluded from the study.

Twenty age- and sex-matched non-leprotic subjects, who were undergoing surgical interventions in Plastic Surgery Department of Ain Shams University Hospitals, were identified as the control group. Informed consent was obtained from all subjects of the study.

Methods. Four millimeter punch skin biopsies were collected from skin lesions of all patients. Skin biopsies of controls were taken from skin adjacent to the surgical wounds. Biopsy specimens from both patients and controls were submerged immediately in 10% buffered formalin, prepared for paraffin embedding and paraffin blocks and sectioned in 4-micron thick sections mounted on glass slides. they were prepared for routine hematoxylin and eosin (H&E) stain for conventional histopathology, and immunohistochemistry for studying the expression of MMP-3 and MMP-9. All slides were coded before analysis and read blindly to ensure competent evaluation of the findings. Conventional H&E sections of patients' biopsies were examined to confirm the diagnosis of leprosy and to categorize the patients into the above-mentioned groups.

Immunohistochemical staining

From each subject, two sections were dried in a 50°C oven for 30 minutes, de-paraffinized in xylene, re-hydrated using a series of alcohols (100%, 90%, and 85%), and washed in PBS (pH 7.4). Endogenous peroxidase was blocked with 0.5% H2O2. Antigen retrieval treatment was performed by boiling in 10 mM citrate buffer solution (pH 6) for 20 minutes followed by cooling at room temperature for 20 minutes. After blocking with normal serum (Lab Vision Corp., Fremont, CA), the slides were incubated with primary monoclonal MMP-3 and MMP-9 rabbit monoclonal antibodies (Lab Vision-cat. RB- 10488-R7-Ready to use, and Lab Vision-cat. RB- 1539-R7- Ready to use respectively) for 32 minutes at room temperature, then with a secondary biotinylated primary antibody, and finally with avidin-biotin-peroxidase complex. Hematoxylin was used as counter-stain. Both MMP-3 and MMP-9 antibody gave brown cytoplasmic immunoreactivity

in positive cases. Positive cases showed heterogeneous pattern and distribution of reactivity differ from one cell type to another (macrophages, lymphocytes, dermal fibroblasts, endothelial cells of blood vessels). Negative staining was considered in absent reactivity (-), and the positive reactivity was graded subjectively as weak (+), moderate (++) or high (+++) according to the intensity of staining. The positive control sections were obtained from placental tissue for each run. Images were captured with a Leica DFC 300 camera.

RESULTS

Conventional H&E sections of patients' biopsies showed epitheliod cells and lymphocytes at the paucibacillary (PB) tuberculoid end of leprosy spectrum, giving way to macrophages which appeared increasingly foamy as the multibacillary (MB) lepromatous pole is reached. In group I, sections showed dermal epithelioid cell granulomas surrounded by a zone of lymphocytes (Figure 1A). In group II, there were diffuse epithelioid cell and foamy histiocyte aggregates with very scanty lymphocytes (Figure 2A). In group III, sections showed foamy macrophages in large aggregates in BL, and in diffuse pattern of dermal infiltrate in LL lesions (Figure 3A). In ENL (group IV), there is evidence of vasculitis with endothelial blood vessels swelling and infiltration of polymorphs inside and around vascular wall (Figure 4A).

The distribution of the immunoreactive MMPs is described in Table 1. Staining intensity varied in proportion to the inflammatory cell number and the cellular composition of the infiltrate.

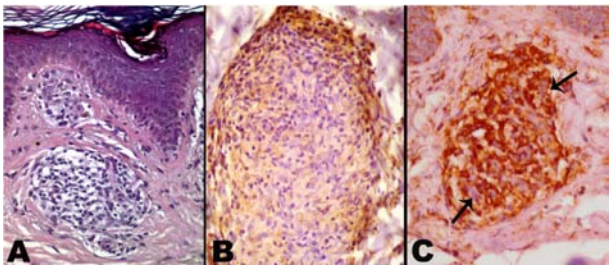

Figure 1. Tuberculoid leprosy **(A)**. A very well-formed epithelioid granuloma surrounded with fibroblasts. (H/E x400) (**B)** Immunoreactivity appears in the fibroblasts coat surrounding the granuloma and in the extracellular matrix. Epithelioid cells are negative to the stain. (Anti MMP-3 x400**) (C)** Immunoreactivity appears strongly (+++) in the epithelioid cells of dermal granuloma (Anti MMP-9 x400).

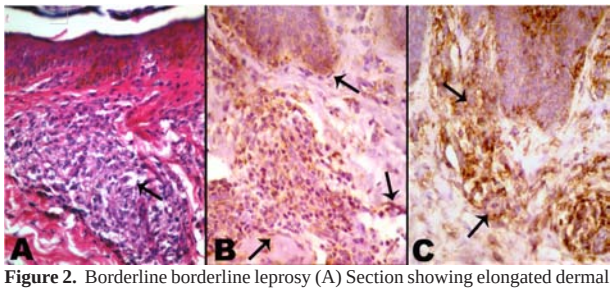

Figure 2. Borderline borderline leprosy (A) Section showing elongated dermal granuloma formed of epithelioid cells and foamy macrophages. (H/E x400) (B) Immunoreactivity appears in fibroblasts and extracellular matrix. (Anti MMP-3 x400). (C) Immunoreactivity appears in the epithelioid cells of dermal granuloma (++) (Anti MMP-9 x400).

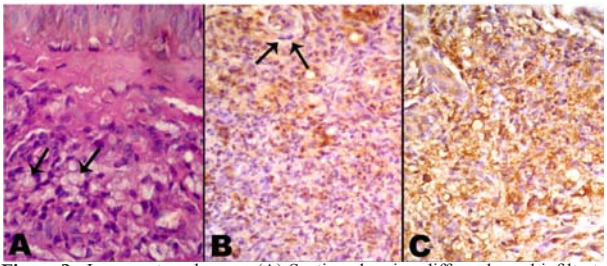

Figure 3. Lepromatous leprosy (A) Section showing diffuse dermal infiltrate formed of foamy macrophages. (H/E x400) (B) Immunoreactivity appears in extracellular matrix and in fibroblasts. The epithelioid cells are negative to the stain. (Anti MMP-3 x400). (C) Immunoreactivity appears moderate in macrophages (++) (Anti MMP-9 x400).

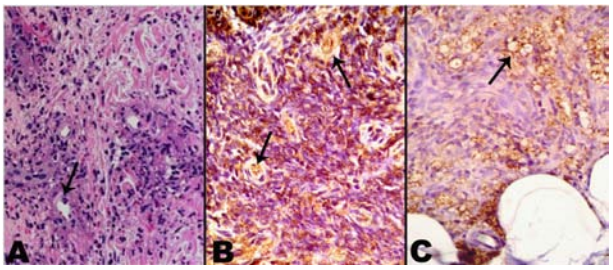

Figure 4. Erythema nodosum leprosum (A) Section of type 2 reaction showing leucocytoclastic vasculitis with dense neutrophilic dermal infiltrate around blood vessels , fibrin depositis, extravasated RBCs ,nuclear dust and endothelial swelling of blood vessel wall. (H/E x400) (B) Immunoreactivity appears strong in endothelial cells of blood vessels (+++), in the surrounding fibroblasts (++) and in the extracellular matrix (+++). The macropgages are negative to the stain. (Anti MMP-3 x400) (C) Immunoreactivity appears in macrophages (+). Endothelial cells are negative to the stain (Anti MMP-9 x400).

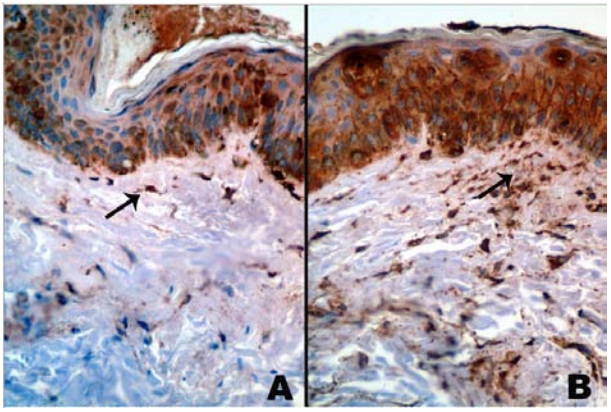

Figure 5. Control skin sections (A) Immunoreactivity appears in keratinocytes and dermal fibroblasts. (Anti MMP-3 x400) (B) Immunoreactivity appears in keratinocytes, dermal fibroblasts and to a lesser extent in macrophages (Anti MMP-9 x400).

Table 1. MMP-3 and MMP-9 immunoreactivity in the different study groups.

Category	MMP-3					MMP-9				
	MQ	L	FB	EN	ECM	MQ	L	FB	EN	ECM
Control	-	-	+	-	-	+	-	+	-	-
TT/BT	-	+	+	-	+	+++	-	+	-	-
BB	-	+	+	-	+	++	-	+	-	-
BL/LL	-	-	+	-	+	+ - ++	-	+	-	-
ENL	-	-	++	+++	+++	+	-	+	-	-

MQ = macrophages, L = lymphocytes, FB = fibroblasts, EN = endothelial cells, ECM = extracellular matrix

Distribution of MMP-3 in different leprosy groups versus controls

In leprosy sections, MMP-3 immunoreactivity was found in fibroblasts, in ECM around cells in ground substance, and in endothelial cells of blood vessels. Some lymphocytes were immunostained. Immunoreactivity was almost nil in TT/BT cases except for some fibroblasts and lymphocytes around epithelioid cell granuloma (Figure 1B). In BB sections positivity was restricted to some fibroblasts and ECM (Figure 2B). In BL/LL, MMP-3 was found in ECM, and fibroblasts, but negative in macrophages (Figure 3B). Immunoreactivity was much increased in ENL at ECM, fibroblasts around sites of vasculitis, and in endothelium of blood vessels, but not in macrophages (Figure 4B).

Normal control sections demonstrated MMP-3 immunoreactivity in keratinocytes and dermal fibroblasts (Figure 5A).

Distribution of MMP-9 in different leprosy groups versus controls

MMP-9 immunoreactivity was most intensely identified in the epithelioid cells of epitheliod granulomas of TT and BT biopsies (Figure 1C) and to a lesser extent in BB biopsies (Figure 2C). Macrophages of BL/LL showed mild to moderate immunoreactivity to MMP-9 (Figure 3C). Although histiocytes were weakly positive, immunostaining was much increased in ENL biopsies in increased number of fibroblasts around vasculitic lesions (Figure 4C).

Normal control sections demonstrated MMP-9 immunoreactivity in keratinocytes, and in dermal fibrobasts and macrophages (Figure 5B).

DISCUSSION

Leprosy is characterized by a wide spectrum of clinical forms, depending on CMI. While in LL there is lack of specific CMI to the causal organism with increased monocyte production of IL-10, and predominant Th2 response with release of different subsets of cytokines (IL-4, IL-5, IL-10, and IL-13), the tuberculoid pole is characterized by Th1 profile with IL-2, IFN-γ, and TNF production[25].

This is the first study to provide evidence of MMPs involvement, and the expression of MMP-3 and MMP-9 in the pathogenesis of different clinical forms of leprosy, according to host immune response. We found that MMP-3 is increased in type 2 lepra reaction; ENL- in fibroblasts, in endothelial cells of blood vessels of vasculitic lesions and in ECM around infiltrating cells, compared to the rest of leprosy groups and to non-leprosy control biopsies.

Erythema nodosum leprosum is a systemic immune-complex reaction with an estimated frequency of 15% among LL and to a lesser extent among BL patients[2]. It may be accompanied by uveitis, dactylitis, arthritis, neuritis, lymphadenitis, myositis, and orchitis[26]. Peripheral neuritis and uveitis with its complications of cataract and glaucoma are the most serious complications of ENL. Attacks are often acute at first but may be prolonged or recurrent over several years, and eventually quiet but insidious, especially in the eye[27]. Corticosteriods and thalidomide are the best known medications for ENL. However, since ENL tends to recur, there is a serious risk of steroid dependency developing in these patients. Also thalidomide, which has a dramatic effect in controlling ENL and preventing recurrences, has serious teratogenic side-effects in early pregnancy, which preclude its use in women of child-bearing age[2], and it is unavailable in several countries, including Egypt. Thus, control of ENL is a real challenge in the disease spectrum of leprosy. Based on our observations, targeted MMP-3 inhibition seems to be promising in this issue.

Recent research has shown that MMPs (including MMP-3) and vascular endothelial growth factor (VEGF) are important in the process of angiogenesis, and that VEGF itself may induce MMP expression, both acting in concert to stimulate angiogenesis[28]. Besides, MMP-3-is involved in leucocyte migration to the sites of inflammation[19], contributing more to the pathogenic changes of immune vasculitis[29]. These findings suggest a role of MMP-3 in angiogenesis and in vasculitis process of ENL.

Angiogenesis, the formation of new blood vessels from the existing vascular bed, has been described as one of the hallmarks of cancer, playing an essential role in tumor growth, invasion, and metastasis[30]. Furthermore, many other diseases are also dependent on upregulated angiogenesis, including vasculitis[4]. When resting endothelial cells are activated by an angiogenic signal, they are stimulated to release degrading enzymes allowing endothelial cells to migrate, proliferate, and finally differentiate to form new vessels[29]. Any of the steps involved in angiogenesis may be a potential target for pharmacological intervention of angiogenesis-dependent diseases. This is the main reason why angiogenesis has attracted recent attention in the field of pharmacological research. Drugs that inhibit MMPs have been recently compared to a mirage, a tantalizing possibility that keeps always out of reach. MMPs are not only involved in the ECM, basement membrane and connective tissue remodeling, but they play a relevant role in the control of other molecules that regulate cell proliferation, migration and differentiation, angiogenesis and apoptosis[28]. The drug BAY12-9566 is a butanoic acid derivative that selectively inhibits MMP-2, MMP-3, and MMP-9. Promising data included inhibition of tumor growth and metastasis in murine melanoma and human colon tumor xenografts, and clinical studies indicated that the compound was relatively well tolerated[30]. Another drug; MMI270 is a potent,

broad spectrum, orally active MMPI that inhibits the gelatinases MMP-2 and MMP-9 and the stromelysin MMP-3 in low nanomolar concentrations[31]. From these studies, it is obvious that increasing number of new anti-angiogenic drugs is entering clinical trials, with exciting new results are awaited in the near future.

The migration of immune cells to sites of infection from the bloodstream requires proteolysis of the basement membrane. In vitro, T cell and dendritic cell migration is in part MMP-9-dependent[32]. In addition to opening a path through the ECM for cell migration, MMP-9 modulates the chemokine and cytokine gradients that drive inflammatory cell recruitment[29]. It also cleaves IL-8 to a fragment with 10 times the potency of the parent molecule[33]. These data demonstrate that MMP-9 activity is required for the normal immune response to infection. Paradoxically, these host-derived enzymes may also cause infection-related immunopathology[5]. While appropriate MMP secretion facilitates an effective immune response, host tissue damage may be caused by excess MMP activity, resulting in matrix catabolism[34]. The balance between matrix catabolism and anabolism is complicated further by the ability of MMPs to degrade non-specific protease inhibitors such as α-1 antiproteinase[35,36], thus swinging the local environment further to matrix breakdown. Tissue destruction may favor pathogen dissemination or persistence, by breaking down barriers to spread or by creating an immunoprivileged site that is poorly accessed by host immune cells[37].

In addition to inducing MMP secretion by host cells, pathogens may further skew the immune response towards tissue destruction by secreting proteolytic enzymes that activate host pro-MMPs. This would represent a biochemically efficient way for the pathogen to cause pathology by operating at the apex of a proteolytic cascade[5]. An example of this is serine proteases associated with LPS preparations that activate MMP-9[38].

In the present study, it was found that increased expression of MMP-9 was mainly observed in functioning macrophages; epithelioid cells of the PB tuberculoid pole of leprosy spectrum and immunoreactivity was decreased towards the MB lepromatous pole, characterized by impaired-functioning foamy histiocytes. However, reactivity was increased in fibroblasts around vasculitis in ENL. These findings indicate that MMP-9 is a key marker of functioning macrophages, epithelioid granuloma formation, and potential destructive effect on peripheral nerves of PB leprotic patients. Besides, it contributes to the inflammation of ENL.

In accordance with these results, Teles et al.[22] were the first to provide evidence of MMP involvement in the pathogenesis of leprosy nerve damage, including MMP-9; an observation that consistently occur with high immune activation in PB leprosy that is closer to the tuberculoid pole of the disease[39], with the configuration of a CD4+ Th1 profile producing higher levels of IFN-γ, TNF-α, and IL-12[40]. It has been reported that nerve lesions in these patients occur earlier than in MB patients, sometimes leading to necrosis of the nerve[39]. Similar changes in MMP-9 expression have been reported in other human neuritis conditions, such as vasculitic neuropathy[41], chronic inflammatory demyelinating polyneuropathy[40] or systemic lupus erythematosus[43] which often results in the chronic, combined axonal, and demyelinating neuropathy. It is worth mentioning that MMP-9 directly contributes to demyelination during peripheral neuropathy, in part by processing myelin basic protein[44].

A striking decrease in the recruitment of macrophages has been reported in MMP-9-depleted animal models of nerve damage by Shubayev et al.[45] implicating this protease in controlling the mechanisms of macrophage migration and/or breakdown of the blood-nerve barrier in experimentally induced Wallerian degeneration. Similarly, previous studies have shown that *M. tuberculosis* infected monocytes, and multinucleate giant cells release functionally unopposed matrix metalloproteinase-9 in vitro and in vivo[6]. Multinucleated giant cells (MGCs) secrete high levels of MMP-9, leading to a blood-nerve barrier breakdown in tuberculous meningitis[46]. In addition, cell wall components of *M. tuberculosis* are capable of inducing gelatinolytic activity of MMP-9 by human monocytes[47].

Recently, MMP-9 was found to be quantitatively the most important of several MMPs secreted by monocytic cells[29]. Active and latent MMP-9 was shown to be inhibited by TIMP-1, the major TIMP secreted by mononuclear phagocytes. MGCs continue to release MMP-9 concentrations, an effect that is not opposed by increased TIMP-1 and that would have the potential to be important in tissue destruction in granuloma[6]. Consistent with this, immunohistochemistry of granulomas from patients infected with *M. tuberculosis* demonstrates extensive MMP-9 staining with minimal TIMP-1 expression despite the presence of only small numbers of bacilli[48]. Thus the high MMP-9 expression detected in PB leprosy patients studied suggests that it contributes to the inflammation, granuloma formation, and nerve destruction taking place in the course of the disease.

The role of MMP-9 in fibrotic diseases of the lung and chronic obstructive pulmonary disease was already shown in many earlier studies[49-53]. Similarly, fibrosis in end-stage neuritic leprosy was postulated to be due to intense degradation and synthesis of ECM proteins during the active neuritic stage, leading to fibrotic endoneurial matrix, with the MMP-9 playing an important role in these processes.

Fibrosis, in turn, impairs nerve fiber regeneration, which explains the irreversibility of the leprosy nerve damage event after the infection has been cured[22]. Therefore, targeting MMP-9 in leprosy could reduce the high disabling potential associated with this disease that interferes dramatically with work and social life of the patient, leading to economic losses and psychological trauma.

To conclude, the evidences outlined above suggest that excess MMP activity may contribute to host damage in leprosy; therefore, the question arises whether modulating MMP activity can improve outcomes. Targeted MMP inhibition is being developed for cancer[30,31,54] and may become a therapeutic option in inflammatory diseases[6]. Broad-spectrum MMP inhibition can be achieved by simple drugs such as tetracyclines, and minocycline, a tetracycline derivative with strong anticollagenase activity, which is proved to inhibit angiogenesis[55]. Furthermore, other MMP inhibitors inhibit the growth of haemangiomas and angiogenesis when injected subcutaneously around tumors[4]. Moreover, MMP-9 activity was specifically reduced in some studies[56] and in combination with MMP3 in others[30,31]. ENL; a potentially hazardous recurrent or chronic immune complex vasculitis in MB leprosy, is a really challenging condition with respect to therapy, that could benefit from targeting MMPs activity. Therefore, host MMP activity can be regulated both by direct inhibitors and by targeting the signaling pathways that up-regulate MMP expression[57]. Natural inhibitors of MMPs do exist, and synthetic inhibitors have been developed which offer the hope of new adjuvant treatment options in leprosy. On the other hand, where bacterial-derived proteases are required for virulence, they are attractive pharmacological targets as they may be inhibited while leaving normal host MMP function intact. Thus, identification and targeting of possible *M. leprae* proteases is another promising potential therapeutic target in leprosy. We hypothesize that regulating excess MMP activity may have benefits in the different clinical forms of leprosy discussed above.

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