Induction of MMP-9 in mouse cerebellum with Purkinje cell loss caused by *Angiostrongylus cantonensis*

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Abstract

Neurological disorders in angiostrongyliasis are caused by invasion of the central nervous system by developing *Angiostrongylus cantonensis* larvae. After *A. cantonensis* infection, the cerebellums of BALB/c strain mice were observed to have a loss of Purkinje cells. Histologically, the Purkinje cells in the infected mice cerebellum were shown to have degenerative atrophy or partial loss. The matrix metalloproteinase-9 (MMP-9) mRNA and activity was induced in the infected mice, and the enzyme was absent in uninfected mice. Western blotting showed that the anti-MMP-9 antibody recognized a single 94 kDa protein from *A. cantonensis*-infected mice. Immunohistochemistry also showed localization of MMP-9 within the degenerative Purkinje cells. These results suggest MMP-9 in mice infected with *A. cantonensis* may be related to the loss of Purkinje cells.

Keywords: *Angiostrongylus cantonensis*, matrix metalloproteinase 9, Purkinje cell

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Running title: MMP-9 in Purkinje cell loss

Introduction

Angiostrongylus cantonensis is a neurotropic nematode whose growth depends on the central nervous system (CNS) of mammalian hosts.[1] Neurological disorders in *A. cantonensis*-infected nonpermissive hosts may thus be ascribed not only to the mechanical damages caused by migrating worms in the brain,[2] but also to the neurotoxicity of eosinophil-derived basic proteins.[3,4] Mice and guinea-pigs infected with *A. cantonensis* exhibit degenerative atrophy or loss of Purkinje cells and spongy vaculation in white matter of the cerebellum.[5]

Matrix metalloproteinases (MMPs) are a family of zinc-binding endopeptidases, capable of degrading various extracellular matrixes (ECM).[6] Excessive cleavage of ECM caused by an imbalance of the MMPs/TIMPs ratio, and excessive proteolysis contributes to various brain pathologies.[7,8,9] In the CNS, MMPs have been implicated in gliomas,[10] neuroinflammation,[11] multiple sclerosis,[12] Alzheimer's disease,[13] Guillain-Barre syndrome,[14] amyotrophic lateral sclerosis,[15] brain trauma and ischemia.[16,17]

While there is some evidence of an important role of MMPs in inflammatory disorders of the CNS,[12,18,19] little is known about their pathological role of MMPs in the CNS. This study used the cerebellum of mice to investigate the expression and activation of MMP-9 using RT-PCR and gelatin zymography. The enzyme was confirmed by Western blot analysis and distribution by immunohistochemistry.

Materials and methods

Experimental animals

The five week old male mice, BALB/c strain, were purchased from the National Animal Breeding and Research Center, Taipei, Taiwan. Mice were maintained at L12 : D12 photoperiod, provided with Purina Laboratory Chow and water *ad libitum*, and kept in our laboratory for more than 1 week before they were infected with *A. cantonensis* for experiment.

Infection of animals

Third-stage (infective) larvae of *A. cantonensis* were obtained from naturally infected giant African snails, *Achatina fulica*, collected from fields in Taichung, central Taiwan. The larvae were liberated from the minced snail tissue by pepsin (Sigma, USA) digestion. Mice were prohibited food and water for 12 h before infection. A total of 20 mice were randomly allocated to control and experimental groups. The control mice were received water and sacrified on day 24 post-infection (PI). The experimental mice were infected with 60 nematode larvae by oral inoculation, and sacrified on day 24 PI.

Histology

The mouse cerebellums were fixed separately in 10% neutral buffered formalin for 24 h. The fixed specimens were dehydrated in a graded ethanol series (50%, 75%, 100%) and xylene, then embedded in paraffin at 55℃ for 24 h. Several serial sections were cut at a 5 μm thickness for each organ from each mouse. Paraffin was removed by heating the sections for 5 min at 65℃. These sections were dewaxed by washing three times for 5 min each in xylene, then rehydrated through 100, 95, and 75% ethanol for 5 min each, and finally rinsed with distilled water. After staining with hematoxylin (Muto, Japan) and eosin (Muto, Japan), pathological changes were examined under a light microscope.

Gelatin zymography

The cerebellar samples were loaded on 7.5% (mass/vol) SDS-polyacrylamide gels that had been co-polymerized with 0.1% gelatin (Sigma, USA).

Stacking gels were 4% (mass/vol) polyacrylamide and did not contain gelatin substrate. Electrophoresis was performed in running buffer (25 mM Tris, 250 mM glycine, 1% SDS) at room temperature at 120 V for 1 h. The gel was washed two times for 30 min each in 2.5% Triton X-100 for 1 h at room temperature, and then washed two times with ddH2O for 10 min each. The gel was incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM CaCl2, 0.02% Brij-35, 0.01% NaN3) at 37℃ for 18 h. The gel was then stained with 0.25% Coomassie brilliant blue R-250 (Sigma, USA) for 1 h, and destained in 15% methanol/7.5% acetic acid. Gelatinase activity was detected as unstained bands on a blue background. Quantitative analysis of the gelatinolytic enzyme was performed with a computer-assisted imaging densitometer system, UN-SCAN-IT$_{TM}$ gel Version 5.1 (Silk Scientific, USA).

SDS-PAGE and Western blot analysis

The mouse cerebellums were homogenized in a buffer containing 0.1% Triton X-100, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM K_2HPO_4. The homogenates were then centrifuged at 12000 g at 4℃ for 10 min, and the protein contents of the supernatants were determined with protein assay kits (Bio-Rad, USA) using bovine serum albumin (BSA) as the standard. An equal volume of loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue) was added to the samples, which contained 30 μg of brain tissue protein. The mixure was boiled for 5 min before being subjected to polyacrylamide gel electrophoresis. Samples were submitted to SDS-polyacrylamide gel under nonreducing conditions and electrotransferred to nitrocellulose membrane at a constant current of 190 mA for 90 min. Afterward, the membrane was saturated with PBS containing 0.1% Tween 20 for 30 min at room temperature. The membrane was allowed to react with goat anti-mouse MMP-9 polyclonal antibody (R&D Systems, USA; 1:100 dilution) at 37℃ for 1 h. Then the membrane was washed three times with PBS containing 0.1% Tween 20 (PBS-T), followed by incubation with HRP-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, USA; 1:5000 dilution) at 37℃ for 1 h to detect the bound primary antibody. The reactive protein was detected by enhanced chemiluminescence (Amersham, UK). To confirm equivalent protein loading, membranes were stripped by incubation in 62.5 mM of Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol at 55℃, subsequently washed with PBS-T, and reprobed with anti-β-actin antibody (dilution 1:500).

RT-PCR analysis of the MMP-9 mRNA levels

The mouse cerebellums were dissected out under a stereoscopic microscope. Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. One microgram of total cerebellar RNA was used for first strand cDNA synthesis in 20 μl of reaction volume using 50 units of Superscript™ II reverse transcriptase (Invitrogen, USA). PCR was performed under standard conditions using Taq DNA polymerase (Invitrogen, USA) and primers. Forward (5'-3') and reverse (5'-3') primers, respectively, were 5'-GACATCAAGAAGGTGGT GAAGC-3'and 5'-TGTCATTGAGAGCAATG CCAGC-3' for GAPDH, 5'-GCGCCACCACA GCCAACTATG-3' and 5' -TGGATGCCGTCT ATGTCGTCTTTA-3' for MMP-9. Identical cycling conditions for GAPDH gene and MMP-9 were a 30 cycle PCR reaction with each cycle consisting of denaturation at 94℃ for 45 sec, annealing at 55℃ for 1 min, primer extension at 72℃ for 2 min, and then holding at 4℃. In

addition, RT-PCR for GAPDH was performed at the same time and under the same conditions. Ten microliters of the amplified product were then subjected to electrophoresis in 1% agarose gels containing 20 μg/ml ethidium bromide in Tris borate-EDTA buffer. Gels were visualized on a UV transilluminator (Taiwan), and digital images were taken using DGIS-5 Digital Gel Image System (Taiwan).

Immunohistochemistry

Ten micrometer paraffin-embedded sections were prepared and mounted on glass slides. Serial sections were deparaffinized with xylene and a graded series of ethanol. Sections were treated with 3% H_2O_2 in methanol for 10 min to inativate endogenous peroxidase, and washed three times with PBS, pH 7.4 for 5 min. Sections were blocked non-specific reactions with 3% BSA at room temperature for 1 h, incubated with goat anti-mouse MMP-9 polyclonal antibody (R&D Systems, USA) diluted 1:50 in 1% BSA at 37℃ for 1 h, and washed three times in PBS for 5 min each. Sections were incubated with HRP-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, USA) diluted 1:100 in 1% BSA at 37℃ for 1 h, and washed three times in PBS for 5 min each. Sections were incubated in DAB (3,3'-diaminobenzidine; 0.3 mg/ml in 100 mM Tris pH 7.5 containing 0.3 μl/ml H_2O_2) at room temperature for 3 min, and washed three times in PBS for 5 min each. Mounted slides with 50% glycerol in PBS were examined under a light microscope.

Results

Histopathological observations

The three zones of the cortex were the basophilic hypercellular granular layer containing numerous small round nuclei, the single cell Purkinje cell layer and the hypocellular molecular layer. While the Purkinje cells in the normal mice were large, pyramidal and were located in the Purkinje cell layer with apical dendrites in the molecular layer (Fig. 1A). Degenerative atrophy and partial loss of Purkinje cells were present in *A. cantonensis*-infected mice (Fig. 1B).

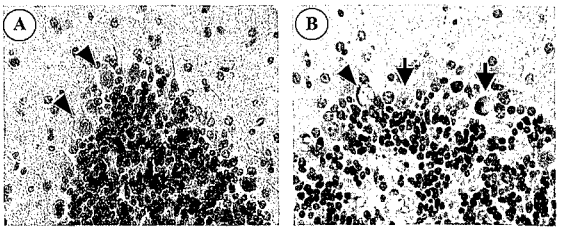

Fig. 1. Histopathological observations. (A) The Purkinje cell layer showing normal morphology and large, pyramidal cells (arrowheads). (B) Purkinje cells in the infected mice *show* degenerative atrophy (arrows) and partial loss (arrowhead) at 24 days PI. Hematoxylin and eosin, X400.

Gelatinase activity assay in mice cerebellum

Analysis of gelatinolytic activity using substrate gelatin zymography showed that the *A. cantonensis*-infected mice presented two bands: one with a lower molecular weight of about 72 kDa, and the other with a higher molecular weight of 94 kDa. The 72 kDa form (gelatinase A, MMP-2) was present in all samples, even in those derived from uninfected mice, whereas the 94 kDa form (gelatinase B, MMP-9) was present only in the samples from mice infected with *A. cantonensis* at 24 days PI (Fig. 2).

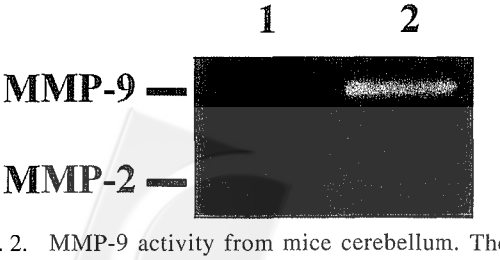

Fig. 2. MMP-9 activity from mice cerebellum. The molecular weight 94 kDa MMP-9 can be detected only in mice infected with *A. cantonensis* at 24 days PI, whereas the 72 kDa MMP-2 was present in all samples.

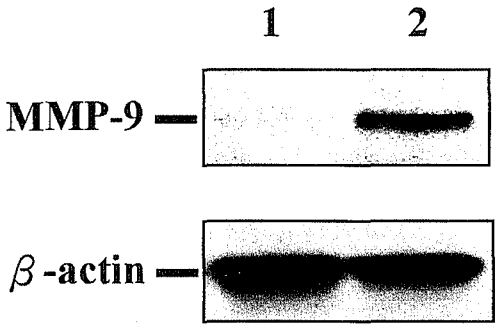

Fig. 3. MMP-9 detection by Western blotting. The latent form of MMP-9 was present in mice infected with *A. cantonensis* at 24 days PI and absent in the uninfected mice. β-actin was performed for a loading control.

Western blotting of the MMP-9

To confirm that the 94 kDa gelatinase was MMP-9, we performed immunoblots of mouse cerebellum homogenates by Western blot analysis. A 94 kDa immunopositive band was detected by anti-MMP-9 antibody in *A. cantonensis*-infected mice at 24 days PI, and it was absent in the uninfected mice, thus indicating that the 94 kDa gelatinase was gelatinase B/MMP-9 (Fig. 3).

MMP-9 mRNA expression in the mouse cerebellum

Total RNA isolated from the mouse cerebellums was assayed for RT-PCR analysis using MMP-9 and GAPDH-specific primers. In order to explore the relationship between MMP-9 and Purkinje cell loss, the expression of MMP-9 in the loss of Purkinje cell was examined. RT-PCR analysis revealed that MMP-9 mRNA was expressed in *A. cantonensis*-infected mice at 24 days PI, and it was absent in the uninfected mice (Fig. 4). Amplified product lengths for GAPDH and MMP-9 were 148 and 798 bp, respectively.

Distribution of MMP-9 in the mouse cerebellum

Because MMPs were the major contributors to ECM degradation, we examined the

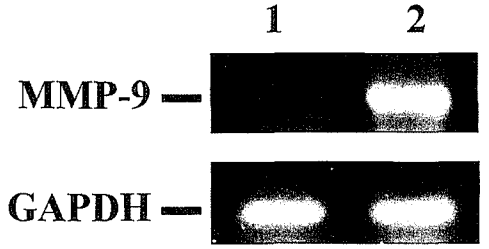

Fig. 4. RT-PCR analysis of MMP-9 mRNA in the mouse cerebellum. (A) The MMP-9 mRNA was expressed in mice infected with *A. cantonensis* at 24 days PI, and was absent in the uninfected mice. GAPDH mRNA was performed for a loading control.

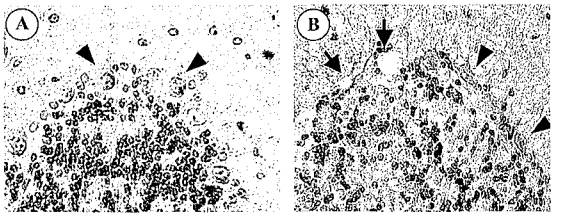

Fig. 5. Distribution of MMP-9 in the mouse cerebellum. (A) No signal can be detected in Purkinje cells of the uninfected mice (arrowheads). (B) MMP-9 was localized in Purkinje cells of *A. cantonensis*-infected mice (arrowheads) at 24 days PI. Arrows indicate the loss of Purkinje cells. Original magnification X400.

localization of MMP-9 in the cerebellum tissue of mice infected with *A. cantonensis* at 24 days PI. Results showed that positive signals for MMP-9 could be localized in cerebellar Purkinje cells (Fig. 5B), and could not be detected in the uninfected mice (Fig. 5A).

Discussion

Purkinje cells in the cerebellum of *A. cantonensis*-infected mice were found by electron microscopic criteria to be degenerative, atrophic and partially depleted.[2,5] In this study, the normal Purkinje cells were large, pyramidal and located in the Purkinje cell layer with apical dendrites in the molecular layer. The cells in the *A. cantonensis*-infected mice were small and irregular, and their loss occurs only in mice infected with *A.*

cantonensis.

The MMP-9 are a family of zinc-dependent enzymes which are able to degrade the protein components of the extracellular matrix.[6] There is accumulating evidence that MMPs and their natural inhibitors play an important role in the pathogenesis of neuroimmunological disease.[20] Changes in MMP expression have also been reported in various neuropathologies involving both neurons (neurodegeneration) and glial cells (inflammation and gliomas).[11] In this study, the expression and activation of MMP-9 were elevated in Purkinje cell loss, however, MMP-9 was not detected within the cerebellum of controls without Purkinje cell loss. The abnormal expression of MMP-9 in the loss of Purkinje cells may have important functional implications.

MMP-9 is involved in the destruction of the basal membrane and degradation of proteins, such as plasminogen activator or β-amyloid, whose activity has been correlated with the progression of neurodegeneration.[21,22] In the present study, the expression of MMP-9 in *A. cantonensis*-infected mice cerebellum was investigated. Upregulation of MMP-9 mRNA was found and associated with increased enzymatic activity and protein immunoreactivity. On immunohistochemistry, positive signals for MMP-9 could be localized in degenerative Purkinje cells. These findings point to a potential role of MMP-9 involved in the degenerative process of Purkinje cells.

Increased MMP expression levels have been observed in several central nervous system disorders. In Alzheimer's disease, MMP-9 has been shown to be present at levels as much as 4-fold higher in the hippocampal region than in age-matched controls.[13] In multiple sclerosis, the level of MMP-9 is also elevated in the cerebrospinal fluid, serum and leukocytes.[11,12] Similarly, the present study was demonstrated that MMP-9 production was markedly enhanced in the cerebellum of *A. cantonensis*-infected mice. However, there was no difference in the levels of MMP-2 band intensities by zymography between infected and uninfected mouse cerebellums. The abnormally high amount of MMP-9 coincides with the time when the Purkinje cell begin to loss and its localization within the degenerative Purkinje cells, together indicate that the enzyme may contribute to Purkinje cell loss by destroying the ECM.

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小白鼠感染廣東住血線蟲誘發基質金屬蛋白酶9與小腦浦金氏細胞喪失之關係

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中文摘要

廣東住血線蟲症(angiostrongyliasis)引起之神經系統疾病是由於發育中的廣東住血線蟲(*Angiostrongylus cantonensis*)侵入中樞神經系統所造成。在感染廣東住血線蟲的BALB/c strain小白鼠發現小腦浦金氏(Purkinje)細胞出現退化性萎縮及部分喪失的現象。基質金屬蛋白酶9(matrix metalloproteinase-9，MMP-9) mRNA的表現及酵素活性只在感染廣東住血線蟲的小白鼠小腦檢測到，經西方點墨法(Western blotting)確定此酵素為MMP-9，而在未感染的小白鼠則檢測不到此酵素。另外，組織免疫試驗亦證實MMP-9定位在退化的浦金氏細胞。這些結果顯示MMP-9可能與小白鼠感染廣東住血線蟲引起小腦浦金氏細胞喪失有關。

關鍵詞：廣東住血線蟲、基質金屬蛋白酶9、浦金氏細胞

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