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Ligation induced Matrix-Metalloproteinase-9 activity in frog peripheral nervous system

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ABSTRACT

Matrix metalloproteinases (MMPs) are endopeptidases that cleave matrix soluble and membrane-bound proteins, and are regulated by their endogenous tissue inhibitors (TIMPs). MMP-2 and MMP-9 are two of the MMPs which contribute to inflammatory and degenerative processes in injured nerves. The aim of the present study was to examine the expression and activities of MMP-2 and MMP-9 in injured and intact frog sciatic nerves using gelatin zymography. As far as we know, our investigation demonstrated for the first time the expression of MMP-2 and MMP-9 in frog sciatic nerves. The expression and activity of MMP-9 were increased twice on average following nerve ligation. By contrast, MMP-2 activity remained unchanged. These findings suggest that MMP-9 can be considered as a marker of degenerative changes that follow nerve ligation in frog nerve.

Key words
Injury • Metalloproteinase • Ligation • Gelatin zymography • Sciatic nerve

Introduction

Damage to the peripheral nervous system (PNS) is followed by Wallerian degeneration (WD) in the nerve distal segment. WD can be induced by experimental nerve crush or section, which promotes a cascade of events described by Waller (1850). The sequence of characteristic events of WD starts with Ca^{+2} influx (Martinez and Ribeiro, 1998) followed by axonal cytoskeleton disintegration and myelin sheath breakdown. After that, macrophages are recruited in the lesion area in order to phagocyte axon and myelin sheath debris (Stoll et al., 1989a; Stoll et al., 2002). All these changes provide specific signals to Schwann cells, which become dedifferentiated and then proliferate forming the bands of Büngner (Stoll and Muller, 1999). The process of regeneration starts with axon sprouting at the nearest node of Ranvier in the nerve proximal stump (Stoll and Muller, 1999). These sprouts achieve the bands of Büngner, and are guided to the target tissue by interacting with Schwann cells basal lamina components. Degradation and remodeling of extracellular matrices are important aspects of the regenerative process.

Matrix-degrading enzymes are expressed not only by regenerating neurons but also by Schwann cells and invading macrophages (Muir, 1994; Muir, 1995; La Fleur et al., 1996). One important class of enzymes involved in the injury-induced remodeling of peripheral nerve are the matrix metalloproteinases (MMPs). The MMPs are a family of zinc-dependent proteinases known for their ability to degrade a wide number of extracellular matrix components. This multigenic family (more than 20 members identified) of zinc-dependent secreted or cell surface-

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associated endopeptidases cleave matrix components and a variety of pericellular proteins, including cytokines, growth factors, cell surface receptors, and adhesion molecules. In addition to their role in matrix dissolution, there is evidence that MMP-mediated matrix degradation can also regulate cell motility and neurite growth (Pittman 1985; Muir, 1994; Giannelli et al., 1997). MMP-2 and MMP-9 (known also as gelatinases) are probably among the most studied of the MMPs in the central nervous system. Unlike MMP-9, which highly inducible in the CNS, MMP-2 is constitutively expressed in neural cells, and can be upregulated in pathological situations (Rivera et al., 1997; Vaillant et al., 1999; Rivera et al., 2002; Szklarczyk et al., 2002; Jourquin et al., 2003; Dzwonek et al., 2004). Proinflammatory cytokines modulate the expression and release of MMP-2, and MMP-9 by neural cells, confirming that these molecules are involved in neuroinflammatory processes (Giraudon et al., 1997; Pagenstecher et al., 1998; Muir et al., 2002; Ogier et al., 2005; Ben-Hur et al., 2006; Sbai et al., 2008). There is also evidence that MMPs play a role in repairing of CNS lesions including myelination or neurogenic migration into damaged tissue (Yong et al., 1998; Rosenberg, 2002).

After transcription and activation, MMPs activity can be regulated by their high affinity tissue inhibitors (TIMPs). These molecules are homologue proteins that can bind to MMPs in a proportion of 1:1 (Bode et al., 1999). Currently, there are four TIMPs described in the literature (1 to 4), and similarly to MMPs, their expression modulates several processes during normal development and pathological conditions (Brew et al., 2000).

The aim of the present study was to examine the activity of two important metalloproteinases: MMP-2 and MMP-9 after nerve ligation in frog sciatic nerves

Material and methods

All experimental procedures were approved by the Ethical Committee and the Animal Care Committee of the Faculty of Bizerte, and were performed according to the Tunisian law on Animal Care Guidelines. All efforts were made to minimize animal suffering and reduce the number of animals used.

Sciatic nerve ligature surgery

Briefly, Frogs (*Rana esculenta*) were anesthetized. The sciatic nerve of right lateral hind limb was exposed, and half of the sciatic nerve was tightly ligated with nylon. As control treatment, called sham, the nerve of left lateral hindlimb of the same frog exposed, but was not subjected to ligation. The sham-operated of the left hindlimb was used as control for each right lateral ligation.

To analyze the time course of MMPs expression after sciatic injury for each point of the time course (0, 3, 6, 9, 10, 12, 15 days), the distal segments of the nerves (6 nerves for each timepoint: 3 ligature nerves and 3 control nerves) were harvested, conserved in Ringer-buffer during 1 to 5 minutes, and analyzed by gel zymography described as below.

Preparation of nerve extracts and protein levels

The sciatic nerves were dissected from their sheaths. Nerve segments were then homogenised and left overnight at 4°C in extraction buffer containing 0.5% Triton X-100, 0.1 M Tris-HCl, pH 8.0. After centrifugation at 15,000 x g for 15 minutes at 4° C, the supernatants transferred to clean eppendorf tubes and stored at -80°C. Proteins concentration were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules CA), based on the Lowry method, using bovine serum albumin as a standard. All data in this study were expressed relative to the protein content.

Gelatin zymography

Nerve extracts containing equal amounts of protein (35 μg) were electrophoresed through an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad) that was polymerized in the presence of 1 mg/ml gelatin. The gels were washed twice for 30 min in 2.5% Triton X-100 to remove the SDS. The gels were then incubated in MMP activating buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.5) at 37°C for 2 days. Gelatinolytic activity was visualized by staining of the gels with 0.1% Coomassie brilliant blue R-250 for 2 hours and destained with a solution containing 5% acetic acid (Sbai et al., 2008).

Data analysis

Zymograms were quantified by densitometry using the Scion Image program. The optical density of

each band was determined in three repeated analyses and averaged together to obtain the data (as plotted in figures. Standard errors of the mean were too small to be illustrated on the graphs).
Statistical analyses was performed using an analysis of variance (ANOVA), followed by Student-*t*-test. Values represent the means ± SEM of 3 independent experiments: *$p < 0.05$.

Results

Endogenous expression levels of gelatinases in frog sciatic nerve

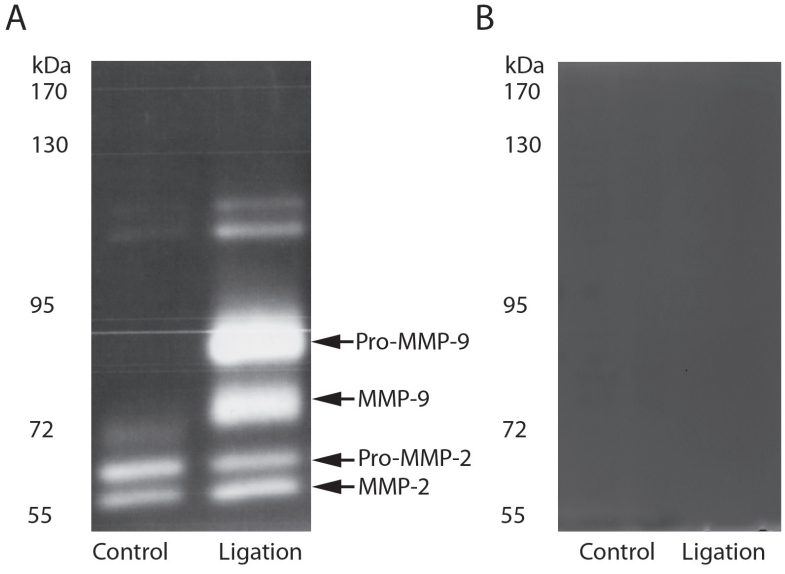
We used gel zymography to analyze the expression of gelatinases in control frog sciatic nerve. MMP-2 was expressed as a doublet with molecular weight of 68 and 58 kDa, corresponding to the pro- and active forms of MMP-2. We observed also two bands at 110 and 120 kDa, maybe resulting from interaction of gelatinases with endogenous protein. MMP-9 was not detected in the Gel zymography (Fig. 1A). The identity of the enzymes as gelatinases was confirmed in the presence of a broad spectrum synthetic inhibitor of MMPs, 1,10-phenanthroline, completely inhibited the gelatinolytic activity of MMP-2 (Fig. 1B).

Effects of ligature on the activity of endogenous gelatinases

After 10 days of nerve ligation, expression of MMP-2 and MMP-9 was assessed. In the ligated sciatic nerves, MMP-9 was expressed as a doublet with molecular weight of 92 and 82 kDa, corresponding to the pro- and active forms of MMP-9 (Fig. 1A). Both untreated and degenerating sciatic nerve lysates showed also the constitutive expression of two bands corresponding to the pro- and active forms of MMP-2. Densitometric scanning of zymograms indicated that only MMP-9 was increased, but not MMP-2 (Fig. 2). The same expressions were evaluated in the proximal nerves: gel zymography showed only the pro-form of MMP-2 (data not show).

MMP expression during Wallerian degeneration in the sciatic nerve

To better characterize the expression of MMPs during Wallerian degeneration, we analyzed the time course of MMP-9 expression after sciatic injury. We observed a significant increase in the expres-



Fig, 1. - Gelatinase expression and activity in control and degenerating sciatic nerves in the absence (A) and presence (B) of broad spectrum MMP inhibitor. (A) Activity at 68 and 58 kDa detected in control sciatic nerve lysates corresponds to the pro- and active forms of MMP-2. In ligated sciatic nerve lysates, there are an additional zones of lysis at 92 kDa and 82 kDa, which correspond to the pro- and active forms of MMP-9. (B) Addition of 1,10-phenanthroline (10 μM), a specific MMP inhibitor, to an identical zymogram run simultaneously confirmed that all the detectable activity is attributable to MMP activity, as the activity was abolished.

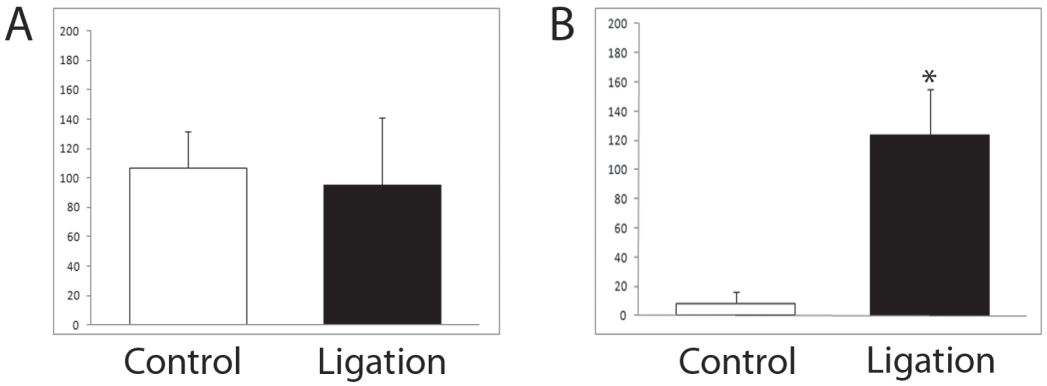


Fig. 2. - Effect of ligation on MMP-2 (A) and MMP-9 (B) in control and after 10 days ligated sciatic nerve.

sion of MMP-9 at day 3 after sciatic nerves injury. Thereafter, MMP-9 expression remained significantly increased until day 10. From 11 days, MMP-9 activity declined, returning to normal at day 15 after injury (Fig. 3).

No change in the expression of MMP-2 were observed in the early or late phase after injury (data not show).

Discussion

The main goal of this study was to investigate the influence of ligation on the expression of gelatinase in frog sciatic nerve. Interestingly, we showed for the first time that MMP-2 and MMP-9 are physiologically expressed in frog sciatic nerve, and MMP-9 expression increases following nerve damage. Thus, MMP expression could be used as a marker of nerve integrity in frog.

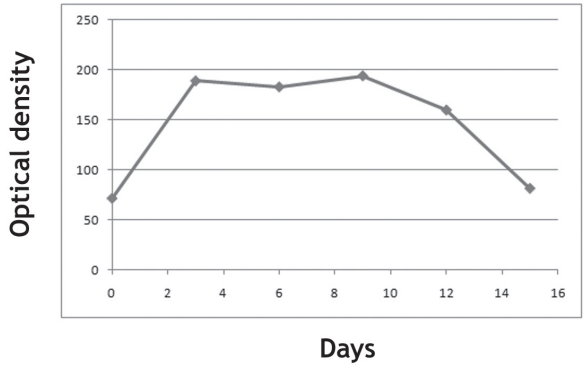


Fig. 3. - Effect of ligation on MMP-9 expression during time. Results are the mean ± S.D. (n = 3 at each time point).

Expression of MMP-2 and MMP-9 in Rana esculenta *sciatic nerve*

This study provides the first evidence of the expression of MMP-2 and MMP-9 in the *Rana esculenta* sciatic nerve. Forty-seven years ago Jerry Gross described the first evidence for MMPs as "a diffusible collagenolytic factor" from bullfrog (*Rana catesbeiana*) tadpole tissues in culture activity while investigating how tadpoles lose their tails. Part of the answer might involve collagenase-1 (MMP1) that Oofusa *et al.* cloned from metamorphosing bullfrog tadpoles. The drastic morphological changes of the tadpole are induced during the metamorphosis, when the concentration of endogenous thyroid hormone is maximal. This systematic reorganization includes the absorption of larva-specific organs such as a tail and gills, the appearance of adult-type organs such as hindlimbs, and the remodeling of intestine, pancreas, brain, skin. The tadpole tail, which is twice as long as the body, shortens rapidly and disappears completely in several days. Many studies showed that MMP genes such as stromelysin-3, collagenase-3 (Brown et al., 1996), and collagenase-4 (Stolow et al., 1996) were induced in thyroid treated tail. Also, thyroid treatment of organ cultured tails augmented MMP-2, MMP-9 and MT3-MMP mRNA (Jung et al. 2002). For these reasons, we hypothesize that MMP-2 and MMP-9 may play a role in tadpole proteolytic activity.

Our data showed that gelatinases were expressed in their pro- and active forms in the sciatic nerve, as MMP-3 in the frog neuromuscular junction (VanSaun et al., 2007). Gelatinases are released in a latent pro-form and have to be activated. The most potent activators of MMP-2 and MMP-9 are plasmin, MMP-1 and MMP-3. Cleavage and subsequent removal of the pro-domain by one of these enzymes releases inhibition of gelatinases catalytic site and access to its substrates.

Ligation induce MMP-9 expression in the frog sciatic nerve

The morphological sequence of events that characterize Wallerian degeneration following nerve ligation in frog sciatic nerve had been well-defined (Stoll et al., 1989a; Stoll et al. 1989b; George and Griffin, 1994; Perry et al., 1995). We demonstrated that after the ligation of frog sciatic nerve the expression of MMP-9 is rapidly induced in the distal segment of the nerve. A similar pattern of mRNA expression, during the early stages of Wallerian degeneration, was also found in the distal segment of the mouse sciatic nerve (La Fleur et al., 1996; Siebert et al., 2001). The localization of MMP-9 to the Schwann cells during this time means that the enzyme could potentially be involved in degradation of the myelin ovoids. Once the fragments of these ovoids have been engulfed by phagocytic cells, lysosomal enzymes are likely to be responsible for further degradation. A recent study showed that in a spinal nerve ligation model, MMP-9 is rapidly produced in injured dorsal root ganglion (DRG) neurons after injury, and induces neuropathic pain at early times through interleukin-1 (IL-1) cleavage, whereas MMP-2 shows a delayed response in DRG neurons inducing late-phase neuropathic pain (Kawasaki et al., 2008). After nerve injury inflammation is an important process that plays a beneficial role in nerve repairing. Macrophage recruitment leads to the rapid clearance of myelin debris and facilitates nerve regeneration (Shubayev and Myers, 2000). In inflammation following nerve injury, MMP-9 plays a role in blood-nerve barrier damage and infiltration of inflammatory cells. MMP-9 null mice had a less-disrupted blood-spinal cord barrier post-injury, with a reduction in neutrophil infiltration (Noble et al., 2002). Actually, inhibition of MMPs might provide a novel therapeutic strategy for the treatment of neuropathic pain.

The activity of most MMPs is regulated at various levels, including transcription, post transcription, and activation of latent forms and the mechanisms for MMPs modulation after nerve injury was still unclear. The rapid upregulation of MMP-9 after nerve injury is thought to be controlled by proinflammatory cytokines TNF-α and IL-1 (Chattopadhyay et al., 2007). MMP-9 levels increased after TNF-α treatment of sciatic nerve *in vivo* and Schwann cells *in vitro*, but this induction was modest compared to its large increase after nerve injury (Shubayev et al., 2006). It indicates that other cytokines or factors might contribute to MMP-9 induction, and that other type of cells in degenerated nerves might also induce MMP-9 after injury.

In this study, we analyzed the effect of ligation on MMP-2 and MMP-9 activities in a frog peripheral nerve. As previously reported, ligation induced MMP-9 at the early phases, but the mechanism of MMP-9 induction at the latter phases was still unclear.

As far as we know, our investigation demonstrates for the first time the expression of MMP-2 and MMP-9 in frog sciatic nerve. The expression and activity of MMP-9 was increased twice on average following ligation. On the contrary, MMP-2 activities remained unchanged. These findings suggest that MMP-9 can be considered as a marker for degenerative changes that follows nerve ligation in frog nerve.

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