Neuroscience 193 (2011) 421–428

DIFFERENT EXPRESSION OF TISSUE INHIBITOR OF METALLOPROTEINASE FAMILY MEMBERS IN RAT DORSAL ROOT GANGLIA AND THEIR CHANGES AFTER PERIPHERAL NERVE INJURY

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Abstract—Matrix metalloproteinase 9 (MMP9) and MMP2 are important in the development and maintenance of neuropathic pain behavior induced by peripheral nerve injury. The enzymatic activity of MMP9 and MMP2 is balanced specifically by tissue inhibitor of metalloproteinase 1 (TIMP1) and TIMP2, respectively. In present study, we measured the effect of peripheral nerve injury on the expression of TIMP1 and TIMP2 in adult dorsal root ganglia (DRG). A dramatic increase of TIMP1 mRNA and a decrease of TIMP2 in DRG after sciatic nerve transection (SNT) were displayed through a real-time PCR method. Furthermore, data showed by *in situ* hybridization that TIMP1 mRNA was only localized in DRG satellite cells under normal conditions. TIMP1 mRNA was increased in satellite cells, and induced within sensory neurons after SNT. Analysis of neuronal profiles showed that induced TIMP1 mRNA was mainly contained in small and medium DRG neurons. Further study displayed that induced TIMP1 mRNA was predominantly present in activating transcription factor 3 (ATF3)-positive injured DRG neurons. Comparatively, TIMP2 mRNA was mostly contained within sensory neurons and the overall amount decreased at the late stage after nerve injury. These data showed different change of TIMPs in DRG after peripheral nerve injury.

Key words: TIMP1, TIMP2, dorsal root ganglia, peripheral nerve injury, *in situ* hybridization.

Peripheral nerve injury could result in neuropathic pain. The multiple pathophysiological changes within injured nerves, primary sensory neurons, and innerved spinal dorsal horns lead to the progress of pain initiation, development, and maintenance (Zimmermann, 2001). Proinflammatory cytokines and chemokines, for example tumor necrosis factor (TNF)-α and interleukin (IL)-1β, play a crucial role in the pathogenesis of peripheral neuropathic pain through peripheral and central mechanisms (Thacker et al., 2007; Leung and Cahill, 2010; Ren and Dubner, 2010). The up-regulation of matrix metalloproteinases (MMPs) was suggested to be involved in the effect of TNF-α and IL-1β (Shubayev and Myers, 2000; Chattopadhyay et al., 2007). Recently, the activation of MMP9 and MMP2 has been showed to be vital to the progress of neuropathic pain at different phases (Kawasaki et al., 2008; Kobayashi et al., 2008).

MMPs are a large family of zinc-dependent extracellular proteases. They can remodel the protein constituents of the extracellular matrix, and process a variety of nonmatrix protein (Page-McCaw et al., 2007). The MMP proteolytic activity is regulated by the interaction with endogenous tissue inhibitors of metalloproteinases (TIMPs) (Brew and Nagase, 2010). To date only four type TIMPs (TIMP1, -2, -3, -4) have been identified. Evidence shows that TIMP1 and TIMP2 have the strong ability to alleviate the nociceptive behavior induced by peripheral nerve injury through the inhibition of the activity of MMP9 and MMP2, respectively (Kawasaki et al., 2008). TIMP1 was reported to be induced in the crush and distal segments of mouse sciatic nerve after injury (La Fleur et al., 1996). And more attentions have been paid to the changes in dorsal root ganglia (DRG) induced by peripheral nerve injury (Ji and Strichartz, 2004). As of this, a particular interest is whether TIMPs are expressed in adult DRG and how their expression levels are regulated by peripheral nerve injury.

EXPERIMENTAL PROCEDURES

Animals

Adult Sprague–Dawley male rats were used (body weight, 200~250 g). The animals were maintained under standard conditions on a 12-h light/dark cycle with water and food *ad libitum*. All animal experiments were approved by the Second Military Medical University Committee on Animal Care and were carried out in adherence with the guidelines of NIH on the use of animal care. All efforts were made to minimize the number of animals used and their suffering.

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Abbreviations: ATF3, activating transcription factor 3; DEPC, diethyl pyrocarbonate; DRG, dorsal root ganglia; GFAP, glial fibrillary acidic protein; IL, interleukin; MMP, matrix metalloproteinase; SNT, sciatic nerve transection; TIMP, tissue inhibitors of metalloproteinase; TNF, tumor necrosis factor.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.07.031

Surgery

Adult rats were anesthetized with sodium pentobarbital (60 mg/kg). In the present study, the sciatic nerve transection (SNT) model was used. Briefly, the left sciatic nerve was exposed at mid-thigh level. The nerve was then strongly ligated and transected distal to the ligation. In all cases, about 5-mm portion of the sciatic nerve was resected distal to the transection. Finally, muscles and skin were sutured in layers, and the animals were allowed to recover from anesthesia in a warmed cage. The rats were allowed to survive for 2, 7, 14, and 28 days after nerve injury.

Real-time PCR

For PCR, L4 and L5 DRGs of rats were dissected and frozen on dry ice. Total RNA from DRGs (L4 and L5) at different time points were extracted with TRIzol reagent (Life Technologies, CA, USA). The RNA (1 μg) of DRGs was reverse transcribed (Superscript II, Invitrogen, Carlsbad, CA, USA) to cDNA. Quantitative PCR was performed with Premix Ex *Taq* (Takara) using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) according to the protocol of the manufacturer. The data were analyzed by 7500 System SDS Software 1.4.0 (Applied Biosystems) using the standard curve method. Expression levels of TIMP1 and TIMP2 were normalized to the values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers for detection of mRNA expression were as following: for TIMP1, 5′CCCAACCCACCCACAGACAG3′ and 5′GCCCGCGATGAGAAACTCCT3′; for TIMP2, 5′GTAGTGATCAGGGCCAAAGC3′ and 5′GTCCCAGGGCACAATAAAGT3′; for GAPDH, 5′CCCAGAACATCATCCCTGCAT3′ and 5′GCATGTCAGATCCACAACGGA3′.

In situ hybridization (ISH)

The rats were anesthetized and perfused via the ascending aorta with saline followed by 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer (pH 7.4). L4 and L5 DRG were then dissected out, and post-fixed in the same fixative for 3 h at 4 °C, and then immersed in 20% sucrose in 0.1 M phosphate buffer for at least 1 day at 4 °C. Diethyl pyrocarbonate (DEPC) water was used for all solutions and appliances necessary for ISH.

A digoxigenin-labeled antisense cRNA riboprobe spanning the entire TIMP1, TIMP2, or TIMP3 coding sequence was generated. DRG sections were fixed in 4% paraformaldehyde for 20 min, treated with proteinase K (10 μg/ml in DEPC water containing 50 mM Tris–HCl, pH 7.5, and 5 mM EDTA) for 20 min, acetylated in 0.25% acetic anhydride/0.1 M triethanolamine (pH 8.0), and prehybridized in hybridization buffer (50% formamide, 5× SSC, 0.3 mg/ml yeast tRNA, 0.1 mg/ml heparin, 1× Denhardt's solution, 0.1% Tween-20, 5 mM EDTA in DEPC water) for 4 h at 65 °C. The prehybridization buffer was substituted by hybridization buffer with 1 μg/ml of the antisense probe in which the sections were incubated for 14 h at 67 °C. After hybridization, excess probe was removed by washing three times with 2× SSC at 67 °C and once with RNase A (1 μg/ml) for 10 min. Sections were then incubated in alkaline phosphatase-conjugated sheep anti-digoxigenin antibodies (1:2000; Roche Molecular Biochemicals), and then in 1 μl/ml NBT and 3.5 μl/ml BCIP substrates in alkaline phosphatase buffer (100 mM Tris–HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20 in distilled water). Control experiments were carried out using a digoxigenin-labeled sense riboprobe for TIMP1, TIMP2, or TIMP3.

For ISH combined with immunohistochemical detection, sections were immunostained with anti-peripherin (rabbit, Millipore, MA, USA), anti-P2X3 (rabbit, Neuromics, MN, USA), anti-glial fibrillary acidic protein (GFAP) (mouse, Millipore, MA, USA), or anti-activating transcription factor 3 (ATF3) polyoclonal antibody (rabbit, Santa Cruz, CA, USA) after ISH process. Images were captured with a Nikon microscope using Neurolucida software.

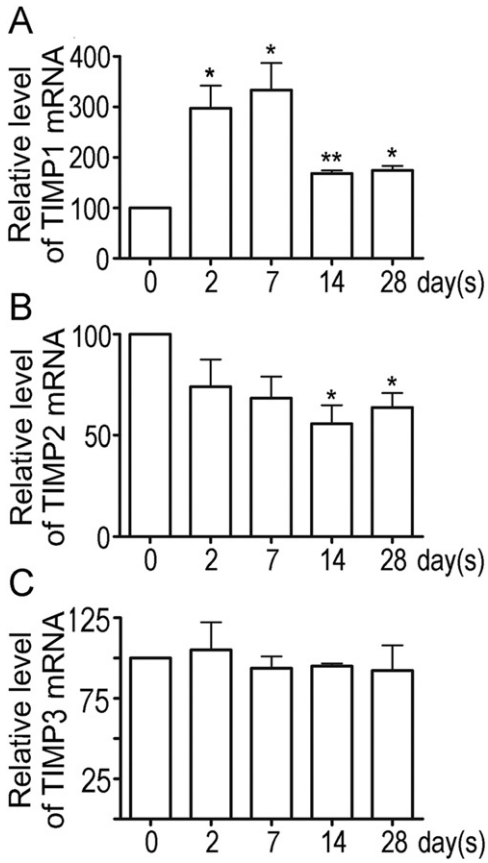

Fig. 1. Change of TIMP1 and TIMP2 mRNA in rat DRG after peripheral nerve injury. (A) PCR shows the increase of TIMP1 mRNA levels in L4 and L5 DRG after SNT. (B) PCR shows the decrease of TIMP2 mRNA levels in L4 and L5 DRG after SNT. (C) PCR shows the expression of TIMP3 mRNA levels in L4 and L5 DRG after SNT. * $P<0.05$, ** $P<0.01$ versus control (day 0).

Quantitative analysis

For quantitative analysis, the data were collected from at least three animals at each time point. To determine the percentage of labeled neuron profiles, the number of positive neuron profiles was divided by the total number of neuron profiles. To determine the distribution of labeled neurons within a subset of DRG neurons, the cross-section area from the neuron profiles with a clear nucleus was examined and indexed with 200-μm^2 interval. Since quantitative analysis of the percentage of the labeled neurons was based on profile counts, it only provided approximate estimates. The data were evaluated by unpaired Student's *t*-test. All data were shown as mean±SEM. $P<0.05$ was considered to be significant.

RESULTS

To identify the effect of peripheral nerve injury on the expression of TIMPs in rat dorsal root ganglia, the levels of TIMP1 and TIMP2 mRNA in the DRG at 2, 7, 14, and 28 days after SNT were measured through a real-time PCR method. Data showed distinct effect of SNT on mRNA level of TIMPs. The expression of TIMP1 mRNA increased noticeably in the DRGs after nerve injury (Fig. 1A). The increased expression reached the ceiling level at day 2