available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Altered expression of Smad family members in injured motor neurons of rat

*Noriko Okuyama, Sumiko Kiryu-Seo, Hiroshi Kiyama**

Department of Anatomy and Neurobiology, Osaka City University, Graduate School of Medicine, 1-4-3 Asahimachi, Abenoku, Osaka 545-8585, Japan

ARTICLE INFO

Article history:
Accepted 6 November 2006
Available online 12 December 2006

Keywords:
TGF-β
BMP
Smad
Regeneration
Nerve injury

ABSTRACT

We examined changes in the expression of *Smad* family members, which transduce signals from TGF-β superfamily ligands, following hypoglossal nerve injury. RT–PCR and *in situ* hybridization revealed that Smad1, 2, 3 and 4 mRNAs were significantly up-regulated in injured side, whereas Smad8 mRNA was down-regulated. Immunohistochemistry and Western blotting analysis confirmed the alterations of Smad1, 2 and 4 in injured neurons. These results suggest that the Smad signaling may be important for nerve regeneration.

Injured neurons in the peripheral nervous system (PNS) are able to survive and regenerate, but those in central nervous system (CNS) are not. In response to peripheral nerve injury, numerous molecules are expressed in neurons, and most of these are assumed to be implicated in regeneration process. Amongst those that have been identified, neurotrophins and cytokines have crucial roles in cell survival and nerve regeneration (Moran and Graeber, 2004; Makwana and Raivich, 2005). It is very intriguing that the receptors for neurotrophins and cytokines, such as TrkA, TrkB, cRet, GFRα1, LIFR and CNTFR-α, are simultaneously induced after nerve injury and contribute effectively to transferring signal transduction (Honma et al., 2002; Makwana and Raivich, 2005). Intracellular signaling molecules such as Shc, ERK, PI3K, Akt, JAKs, Tyk and STAT3, which act downstream of those receptors, are also up-regulated after nerve injury (Kiryu et al., 1995a,b; Yao et al., 1997; Tanabe et al., 1998; Namikawa et al., 2000; Snider et al., 2002; Makwana and Raivich, 2005). Therefore, the orchestrated inductions of both receptors and their downstream signaling molecules are perhaps important for proper regeneration.

In addition to those protective molecules, TGF-β superfamily members, including transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP) and activin, are putative protective factors (Kiefer et al., 1993; Jiang et al., 2000). TGF-β family members bind to type I and type II serine/threonine kinase receptors and activate intracellular Smad proteins, which are key mediators for TGF-β family signaling (Miyazawa et al., 2002; Ten Dijke and Hill, 2004). Smads are classified into three subclasses based on their structure and function. The first class, called receptor-regulated Smads (R-Smads), includes Smads 1, 2, 3, 5 and 8. This class can be further divided into two categories: Smad2 and Smad3 respond to TGF-β and activins, whereas Smads 1, 5 and 8 function in BMP signaling pathway. R-Smads are directly

* *Corresponding author*. Fax: +81 666 45 3702.
E-mail address: kiyama@med.osaka-cu.ac.jp (H. Kiyama).

0006-8993/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2006.11.019

phosphorylated and activated by the type I serine/threonine kinase receptor. The second class is the common partner Smad (Co-Smad), which contains only Smad4. R-Smads oligomerize with Co-Smad to form a heterodimeric complex that is translocated into the nucleus where it modulates transcriptional responses. The third class, called inhibitory Smads (I-Smads), includes Smads 6 and 7. They act as inhibitors of Smad-mediated signal transduction by interacting with the type I receptor and preventing activation of R-Smads (Miyazawa et al., 2002). It is therefore critical to understand whether Smad-mediated signaling is involved in nerve regeneration. However, little is known of the alterations of Smad proteins in response to nerve injury. In this study, we examined the mRNA expression and protein levels of Smad family members using a hypoglossal nerve injury model of rat.

Male Wistar rats (7 weeks old, 42 rats) were anesthetized with pentobarbital (45 mg/kg, i.p.) and placed in a supine position. Their right hypoglossal nerve was cut just proximal to the bifurcation of the nerve. For reverse transcriptase polymerase chain reaction (RT–PCR), five rats were killed by decapitation 7 days after hypoglassal nerve injury. Control and injured hypoglossal nuclei were dissected under a microscope and quickly dipped into liquid nitrogen. Total RNA was obtained from the control and injured hypoglossal nuclei, and reverse-transcribed with oligo dT using Super-Script II (Invitrogen) according to the manufacturer's protocol. RT–PCR was performed using following specific primers for Smads 1–8: Smad1 sense 5′-TTGTTTAGAAATGAATGGGTT-3′, Smad1 antisense 5′-ACAGTTAGAGGAATTAACCAGCTG-3′, Smad2 sense 5′-CAGCTTCTCTGAACAAACCAGG-3′, Smad2 antisense 5′-TACTCTGTGGCTCAATTCCTGCTG-3′, Smad3 sense 5′-TGACAGTGCTATTTTCGTCCAGTCT-3′, Smad3 antisense 5′-CGATCCCTTTACTCCCAGTGTCT-3′, Smad4 sense 5′-TGTCTCACCTGGAATTGATCTCTCAG-3′, Smad4 antisense 5′-AATCCATTCTGCTGCTGTCCTGGCTG-3′, Smad5 sense 5′-CAGATGGGCTCTCCGCTGAACC-3′, Smad5 antisense 5′-TCGT TTACAATACTTTTGAAAG-3′, Smad6 sense 5′-ATGAC-CAGGCTGTCAGCATCTTCTA-3′, Smad6 antisense 5′-ATCTGTGGTTGTTGAGGAGGATCT-3′, Smad7 sense 5′-TCA-GATTCCCAACTTCTTCTGGAGCC-3′, Smad7 antisense 5′-TGT GAAGATGACCTCCAGCCAGCAC-3′, Smad8 sense 5′-AGCACCCCCTGCTGGAT-3′ and Smad8 antisense 5′-AAG-TAGGTAGCACAGAAC-3′. RT–PCR was performed by 23 to 32 cycles of PCR depending on the target genes, with annealing temperatures 60 °C. Products were separated on an agarose gel and visualized using ethidium bromide. For *in situ* hybridization, brains were removed quickly 7 days after axotomy and frozen in powdered dry ice. Sections were cut at a thickness of 15 μm using a cryostat. All procedures for *in situ* hybridization were performed as described previously (Kiryu et al., 1995a,b). Data are representative of three independent experiments using at least seven animals. For immunohistochemistry, brains (n=20) were removed 7 days after hypoglossal nerve injury and divided into two groups; one for fresh–frozen brain preparation and the other for perfusion fixation with Zamboni's fixative (picric acid/paraformaldehyde). 15 μm sections were cut using a cryostat, thaw-mounted onto 3-aminopropyltriethoxysilane-coated slides. For Smad1 immunoreactivity, fresh-frozen sections were fixed in 2% paraformaldehyde, permealized for 10 min in 1% Triton X-100 in TBS (20 mM Tris–HCl, 136 mM NaCl), blocked with 1% bovine serum albumin and incubated with anti-Smad1 antibody (sc-7965, Santa Cruz). For Smad2/3 immunoreaction, Zamboni-fixed section was used. The sections were permealized for 10 min in 1% Triton X-100 in TBS (20 mM Tris–HCl, 136 mM NaCl), blocked with 1% bovine serum albumin and incubated with anti-Smad2/3 antibody (sc-6033, Santa Cruz). For Smad4 immunoreaction, fresh-frozen sections were fixed. To retrieve antigen, sections were boiled in a microwave in 10 mM citrate buffer for 20 min, permealized for 10 min in 1% Triton X-100 in TBS (20 mM Tris–HCl, 136 mM NaCl), blocked with 1% bovine serum albumin and then incubated with anti-Smad4 antibody (sc-7966, Santa Cruz). After incubation with the antibodies at 4 °C overnight, sections were incubated in secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes) for 1 h at room temperature. For Western blotting, samples collected from control and injured hypoglossal nuclei of five rats 7 days after axotomy were homogenized in lysis buffer (8 M Urea, 2% CHAPS, 40 mM Tris, 1 mM Na_3VO_4, 10 mM NaF). The homogenate was centrifuged at 4 °C for 10 min at 14,000 rpm. 20 μg of supernatant was loaded and immunoblotted with anti-Smad1 (sc-7965, Santa Cruz), anti-Smad2/3 (sc-6033, Santa Cruz), anti-Smad4 (sc-7966, Santa Cruz) and anti-GAPDH (#4300, Ambion), visualized with horseradish peroxidase-conjugated secondary antibodies using electrochemiluminescence (Perkin-Elmer).

To clarify whether Smad family members are involved in nerve regeneration, we examined the expression profiles of Smad members such as Smads 1, 2, 3, 4, 5, 6, 7 and 8 after hypoglossal nerve injury. We initially performed RT–PCR analysis using mRNAs isolated from control and injured hypoglossal nuclei. Among the various Smad family members, mRNAs for Smad1–4 were significantly increased after axotomy, whereas that for Smad8 was decreased. No significant changes in mRNA expression were observed in Smads 5, 6, and 7 (Fig. 1). To confirm these alterations and further identify the cell types expressing those members, *in situ* hybridization was carried out. *In situ* hybridization showed that the expression of Smad1, 2, 3 and 4 mRNAs was markedly up-regulated on the injured side 7 days after hypoglossal nerve transection, whereas that of Smad8 mRNA was down-regulated (Fig. 2A). The expression levels of Smad5, 6 and 7 mRNAs were very low and the apparent alterations of mRNAs were not detected. To semi-quantify the fold increase of mRNA expression after axotomy, we measured the signal intensity on the film-autoradiogram shown in Fig. 2A. Among the Smad family members examined, mRNAs for Smads 1–4 were significantly up-regulated by 3- to 7-fold following nerve injury. In contrast, Smad8 mRNA was down-regulated by 4-fold after axotomy (Fig. 2B). These results were consistent with those of RT–PCR. To clarify the cell types expressing Smad mRNAs, an emulsion autoradiogram was observed under bright-field illumination after Nissl staining. The hybridization signals for Smads 1, 2 and 4 were accumulated on large neurons but not in the surrounding glial cells (Fig. 2C arrows), whereas that of Smad3 was not seen on large neurons suggesting that it was expressed in non-neuronal cells (Fig. 2C arrow heads). Smad8 mRNA signal was also observed in