Experimental Neurology 250 (2013) 353–365

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Smad3 deficiency increases cortical and hippocampal neuronal loss following traumatic brain injury

Sonia Villapol [a,b,1], Yu Wang [a,c,1], Matthew Adams [a], Aviva J. Symes [a,b,*]

[a] Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA
[b] Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA
[c] Department of Neurology, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, China

ARTICLE INFO

Article history:
Received 9 June 2013
Revised 11 September 2013
Accepted 3 October 2013
Available online 9 October 2013

Keywords:
TGF-β
TβRII
Smad3
pSmad3
Neurodegeneration
Neuron
Astrocyte
Cell death
CA1
Caspase-3

ABSTRACT

Transforming growth factor-β (TGF-β) signaling is involved in pathological processes following brain injury. TGF-β signaling through Smad3 contributes significantly to the immune response and glial scar formation after brain injury. However, TGF-β is also neuroprotective, suggesting that Smad3 signaling may also be involved in neuroprotection after injury. We found expression of the TGF-β type II receptor (TβRII) and Smad3 protein to be strongly and rapidly induced in neurons in the ipsilateral cortex and CA1 region of the hippocampus after stab wound injury. In contrast, astrocytic expression of TβRII and Smad3 was induced more slowly. Comparison of the response of wild-type and Smad3 null mice to cortical stab wound injury showed a more pronounced loss of neuronal viability in Smad3 null mice. Neuronal density was more strongly reduced in Smad3 null mice than in wild-type mice at 1 and 3 days post lesion in both the ipsilateral cortex and hippocampal CA1 region. Fluoro-Jade B, TUNEL staining, and cleaved caspase-3 staining also demonstrated increased neuronal degeneration at early time points after injury in the ipsilateral hemisphere in Smad3 null mice. Taken together, our results suggest that TGF-β cytokine family signaling through Smad3 protects neurons in the damaged cortex and hippocampus at early time points after injury.

Published by Elsevier Inc.

Introduction

Transforming growth factor-β (TGF-β) is widely recognized as a critical regulator of key events in development, disease, and repair (Dunker and Krieglstein, 2000; Massague, 1998). TGF-β regulates inflammatory responses after injury in the peripheral and central nervous system (CNS) (Wyss-Coray et al., 1997), and functions as a survival factor for embryonic motoneurons, dopaminergic and neonatal sensory neurons in vitro (Chalazonitis et al., 1992; Krieglstein et al., 1995; Martinou et al., 1990; Poulsen et al., 1994). TGF-β exerts neurotrophic effects on damaged neurons (Prehn et al., 1993), rescues sympathetic neurons from death after destruction of the target cells (Blottner et al., 1996), and protects dopaminergic neurons from *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity and cortical neurons from sodium cyanide or glutamate (Farkas et al., 2003; Krieglstein et al., 1995; Prehn et al., 1993). Further, antagonism of TGF-β receptor signaling led to increased neurodegeneration in models of either excitotoxic injury or Alzheimer's disease (Ruocco et al., 1999; Tesseur et al., 2006). Thus, TGF-β signaling is critical for the brain injury/repair response under a variety of different conditions.

TGF-β is induced after traumatic brain injury, and may act as a neuroprotective factor in the injured CNS (Lindholm et al., 1992; Logan et al., 1999b; Stoll et al., 2004). However, after brain injury TGF-β is also responsible for deposition of extracellular matrix proteins that contribute to glial scar formation (Logan et al., 1994, 1999a) which retards neuronal regeneration (McKeon et al., 1995; Windle et al., 1952) and increases the induction of traumatic epilepsy after brain injury (Hoeppner and Morrell, 1986; Tian et al., 2005). Additionally, TGF-β may suppress the early stages of neurogenesis (Buckwalter et al., 2006; Wachs et al., 2006), which may reduce the regenerative capacity of the brain. Thus, targeting TGF-β to enhance recovery from traumatic injury becomes complex because of these multiple and sometimes conflicting functions. It is therefore critical to identify the TGF-β transduction pathways and downstream effectors through which TGF-β mediates individual effects in an effort to target specific pathways mediating detrimental effects while leaving its beneficial effects unaltered.

Active TGF-β induces the formation of receptor hetero-tetramers that contain two "type I" (TβR-I) and two "type II" (TβR-II) transmembrane serine threonine kinase receptors (Vilar et al., 2006). TGF-β isoforms elicit their specific responses through the ligand-induced formation of a heteromeric receptor complex between TβR-I and TβR-II. TβR-II

* Corresponding author at: Department of Pharmacology, Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD 20814, USA. Fax: +1 301 295 3220.
E-mail address: aviva.symes@usuhs.edu (A.J. Symes).
[1] These authors contributed equally to this manuscript.

0014-4886/$ – see front matter. Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.expneurol.2013.10.008

binds TGF-β first, and then recruits TβR-I, allowing for the transphosphorylation-mediated activation of TβR-I. This ligand–receptor activation leads to phosphorylation of Smad2 and Smad3 by the type I receptor, and their release from a cytoplasmic protein (reviewed in (Derynck and Zhang, 2003; Heldin et al., 1997; Krieglstein et al., 1995). These receptors activate Smad proteins (R-Smads) which then form heteromeric complexes with the pathway-independent Smad4 (Lagna et al., 1996). The Smad complex translocates to the nucleus where it regulates transcription of TGF-β target genes (Massague et al., 2005). Smads interact with a diverse array of transcription factors to bring about TGF-β regulated transcription (Feng and Derynck, 2005; ten Dijke and Hill, 2004). However, although Smad proteins are critical to many of the actions of TGF-β, TGF-β utilizes several different pathways for signaling within cells, including Smad-independent pathways (Farkas et al., 2003; Massague, 2003). Indeed, it has been proposed that the neuroprotective effects of TGF-β may be transduced by the alternative TβR-I, Alk1 signaling through NF-κB (Konig et al., 2005).

Smad proteins are involved in many of the same neuroprotective or neurodegenerative processes that have been attributed to TGF-β, suggesting that TGF-β signaling through Smad2 or Smad3 is critical for many of these effects. Elevation of TGF-β levels after traumatic brain injury correlate with local and rapid activation of Smad2/3-dependent signaling in the lesioned brain (Lin et al., 2005). Activation of Smad3-dependent signaling in astrocytes protects neurons against NMDA-induced cell death (Docagne et al., 2002). Studies on Smad3 deficient mice have revealed a role for Smad3 in trophic support for nigral dopaminergic neurons (Tapia-Gonzalez et al., 2011), in addition to maintenance of neurogenesis and migration along the rostral migratory stream to the olfactory bulb (Wang and Symes, 2010). Dysregulation of TGF-β-Smad signaling by Tau hyperphosphorylation may contribute to the neurodegeneration inherent in Alzheimer's disease (Baig et al., 2009; Ueberham et al., 2006).

Our previous study showed that Smad3 deficient mice had reduced scar formation and less immune infiltration after a traumatic brain injury, suggesting a beneficial effect of Smad3 deficiency on recovery (Wang et al., 2007). However, it remains unknown if Smad3 plays a role in neuronal survival after traumatic brain injury. In the present study, we used these Smad3 deficient mice to investigate the potential neuroprotective role of Smad3 in the damaged cortex and hippocampus after cortical stab wound injury.

Materials and methods

Animals

Mice homozygous for a targeted deletion in exon 8 of the Smad3 gene ($Smad3^{ex8/ex8}$) and their wild-type littermates were bred from heterozygous $Smad3^{ex8/+}$ mice on a mixed C57BL/6 × SV129 background. All experiments were performed on mice between 2 and 3 months old. Genotype was determined by PCR analysis of genomic DNA isolated from tail clippings at 3 weeks of age. Presence of the wild-type *Smad3* allele was established using primer 1: 5′-cca ctt cat tgc cat atg ccc tg-3′ with primer 2: 5′-ccc gaa cag ttg gat tca cac-3′. The targeted deletion was detected with primer 1 and primer 3: 5′-cca gac tgc ctt ggg aaa agc-3′ (Yang et al., 1999). The protocols in this study were approved by the USUHS IACUC and were in accordance with the animal welfare guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Stab injury

Cortical stab wound injury was carried out as previously described (Wang et al., 2007). Briefly, mice were anesthetized with ketamine/xylazine solution (50 mg/kg ketamine/7.5 mg/kg xylazine in 0.9% NaCl solution) and placed in a stereotaxic frame. A 1.5-mm grove was drilled in the cranium 2 mm caudal to the Bregma, 1 mm lateral to the midline. A 1.1-mm sterile sharp knife was inserted vertically into the right cerebral hemisphere 2 mm deep to the dura surface. The knife was left in situ for 1 min. The injury site was covered with sterile bone wax; the skin incision was closed with sutures. At different times (1, 3, 7 or 14 days post lesion (dpl)) mice were deeply anesthetized then perfused with ice-cold fresh 4% paraformaldehyde solution.

Preparation of tissue

Brains were fixed in 4% paraformaldehyde for 24 h at 4 °C, cryoprotected in 10%, 20% and 30% sucrose for 24 h individually, then quick frozen on dry ice and stored at −80 °C. 10 μm coronal sections were cut sequentially through the entire lesion site on a cryostat, mounted on gelatin coated glass slides and stored at −80 °C until use. Six sections spaced 100 μm apart from each other through the width of the lesion were mounted on each slide to provide evenly distributed sections for each antibody stain.

Fluorescence immunohistochemistry

Sections taken from the −80 °C freezer were washed with 0.1% Triton X-100 in 0.1 M phosphate buffered saline (PBST), blocked in PBST/10% normal goat serum (NGS) for 1 h, then incubated overnight at 4 °C in PBST/1% NGS containing the primary antibody. Sections were then washed in PBST three times and incubated with the corresponding secondary antibodies for 2 h at room temperature. For double labeling of Smad3/NeuN and Smad3/GFAP, the two primary antibodies were incubated simultaneously with the sections, as were the correspondent secondary antibodies after washing with PBST. Sections were rinsed once with PBST followed by a distilled water rinse before coverslipping with ProLong Gold antifade reagent (Invitrogen). The following primary antibodies were used: anti-NeuN (mouse monoclonal, 1:200, Millipore); anti-GFAP (mouse monoclonal, 1:1000; Millipore); anti-TβRII (rabbit polyclonal, 1:15,000; Santa Cruz Biotech); anti-Smad3 (rabbit polyclonal, 1:100; Upstate); and anti-pSmad3 (rabbit polyclonal, 1:1000, Cell Signaling). Secondary antisera were either anti-mouse or anti-rabbit IgG conjugated to Alexa Fluor 488 or Alexa Fluor 568 (1:1000, Invitrogen). Sections were also incubated with secondary antisera alone to control for non-specific binding of the secondary antiserum to the mouse brain sections. For costaining of Smad3/TβRII and pSmad3/TβRII, staining was performed sequentially, as both primary antibodies were raised in rabbit. Sections were blocked overnight in PBST/10% NGS before overnight incubation with the first primary antiserum in PBST/1% NGS. After washing, sections were incubated with the corresponding secondary antiserum for 2 h, before blocking again (PBST/10% NGS) overnight to avoid cross-reactivity. Incubation with the second primary antiserum was again performed overnight in PBS/1% NGS before a 2 h incubation with the second secondary. Sections were washed with PBS followed by rinsing in distilled water before coverslipping with ProLong Gold antifade reagent. Images (20×) were acquired using an Olympus BX61 fluorescence microscope attached to a Retiga EXi Aqua CCD camera (Qimaging), using iVision software (BioVision Technologies). For colocalization studies, double-stained cells were analyzed using a Zeiss confocal-laser scanning microscope (LSM 510) equipped with argon and He/Ne laser emitting at 488 nm and 568 nm.

Assessment of cell damage

Fluoro-Jade B histofluorescence was performed as described (Schmued et al., 1997). Briefly, brain sections were removed from the −80 °C and left at room temperature for 20 min. Sections were sequentially immersed in 100% ethyl alcohol (3 min), 70% alcohol (1 min) then water (1 min). They were then immersed in 0.06% potassium permanganate for 15 min while shaking gently. After a distilled water rinse they were transferred to the 0.001% Fluoro-Jade B staining solution for 30 min. Sections were rinsed three times with