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Full-length Article

Transforming growth factor-beta 1 signaling regulates neuroinflammation and apoptosis in mild traumatic brain injury

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ABSTRACT

Mild traumatic brain injury (mTBI) is a low-level injury, which often remains undiagnosed, and in most cases it leads to death and disability as it advances as secondary injury. Therefore, it is important to study the underlying signaling mechanisms of mTBI-associated neurological ailments. While transforming growth factor-beta1 (TGF-β1) has a significant role in inflammation and apoptosis in myriads of other pathophysiological conditions, the precise function of increased TGF-β1 after mTBI is unknown. In this study, our objective is to study the physiological relevance and associated mechanisms of TGF-β1-mediated inflammation and apoptosis in mTBI. Using an *in vitro* stretch-injury model in rat neuronal cultures and the *in vivo* fluid percussion injury (FPI) model in rats, we explored the significance of TGF-β1 activation in mTBI. Our study demonstrated that the activation of TGF-β1 in mTBI correlated with the induction of free radical generating enzyme NADPH oxidase 1 (NOX1). Further, using TGF-β type I receptor (TGF-βRI) inhibitor SB431542 and transfection of TGF-β1 siRNA and TGF-β antagonist Smad7, we established the neuroinflammatory and apoptotic role of TGF-β1 in mTBI. Inhibition of TGF-βRI or TGF-β1 diminished TGF-β1-induced inflammation and apoptosis. Further, the enhanced TGF-β1 activation increased the phosphorylation of R-Smads including Smad2 and Smad3 proteins. By immunofluorescence, western blotting, ELISA and TUNEL experiments, we demonstrated the up-regulation of pro-inflammatory cytokines IL-1β and TNF-α and apoptotic cell death in neurons. In conclusion, this study could establish the significance of TGF-β1 in transforming the pathophysiology of mTBI into secondary injury.

1. Introduction

Traumatic brain injury (TBI) occurs when an external force traumatically injures the brain; this often leads to physical, cognitive and/or emotional deficits (Teasdale and Jennett, 1974; del Zoppo and Mabuchi, 2003; Chen et al., 2011). Based on the severity of the injury, the TBI patients are typically categorized into mild, moderate, or severe by the Glasgow Coma Scale (Teasdale and Jennett, 1974). TBI with a low level of injury is called mild TBI (mTBI), and it comprises ~80% of all TBI cases in the US. In mTBI, there is no visible brain damage, but the trauma causes changes in the pathophysiology of brain and associated organs/systems, sometimes leading to death (Goldstein et al., 2012; Abdul-Muneer et al., 2013). Mild injuries often remain undiagnosed until they advance as secondary injuries, expressed as long-term neurological deficits (Alexander, 1995; Abdul-Muneer et al., 2013). mTBI is known to trigger inflammatory cascades by increasing the level of cytokines and chemokines, which result in monocyte/leukocyte activation and infiltration, glial activation, neuronal and myelin loss and ultimately long-term neurological deficits (Shlosberg et al., 2010). Therefore, it is important to elucidate the underlying signaling mechanisms of mTBI-associated neurological ailments that have paramount importance for developing therapeutic interventions to treat TBI patients.

In previous studies, we have established that oxidative and nitrosative radicals play critical roles in the progression of brain injury in mTBI (Abdul-Muneer et al., 2013, 2015a,b, 2016, 2017). We demonstrated the activation of matrix metalloproteinases (MMPs), disruption of blood–brain barrier (BBB), and induction of inflammatory signaling as secondary effector mechanisms of TBI-mediated free radical accumulation in a rat model of mTBI (Abdul-Muneer et al., 2013). Activated MMPs by oxidative stress cleave extracellular matrix and tight junction proteins that impair brain endothelium stability, thereby compromising the BBB (Abdul-Muneer et al., 2013). Recently, we demonstrated that oxidative stress-induced MMP2 cleaves stromal cell derived factor

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1 α (SDF-1α) to a neurotoxic fragment called SDF-1(5-67) and contributes to neurodegeneration (Abdul-Muneer et al., 2017). The study explained the role of free radical generating enzyme NADPH oxidase 1 (NOX1) and the corresponding marker of the oxidative damage 4-hydroxynonenal (4HNE) in activating MMP2 (Abdul-Muneer et al., 2017). Based on these studies, it is evident that oxidative signaling is the central mechanism responsible for TBI associated neurological impairments. Since oxidative stress is linked to the activation of transforming growth factor-β1 (TGF-β1), inflammation, and apoptosis (Jain et al., 2013; Krstic et al., 2015), in this study, we hypothesize that TGF-β1 may also be involved in the activation of TBI-induced neuroinflammation and neurodegeneration.

TGF-β1 is a member of TGF-β super family of cytokines, which functions in growth, development, immunity, wound healing, inflammation, apoptosis, and cancer (Blobe et al., 2000; Wu and Hill, 2009). TGF-β signaling initiates as the ligand binds to the receptors followed by the dimerization of type I and type II receptors (Massague, 2012). The activation of the type I receptor is subsequently followed by the phosphorylation of Smad (Mothers against decapentaplegic homolog) proteins including Smad2, Smad3, and Smad4, which then translocates into the nucleus and regulates the transcription of related genes (Eppert et al., 1996; Blobe et al., 2000; Miyazono et al., 2000). Though few authors reported TGF-β as a neuroprotective factor depending on the signaling pathways in different environmental conditions (Vogel et al., 2010; Logan et al., 2013), its role as excitotoxic and inflammatory factor is well established in different pathophysiological conditions (Lindholm et al., 1992; Luo et al., 2006; Town et al., 2008; Lanz et al., 2010; Manaenko et al., 2014; Rustenhoven et al., 2016). Up-regulation of TGF-β has been noticed during different pathological conditions such as multiple sclerosis, stroke, Alzheimer's disease, tumors, and trauma (Krupinski et al., 1996; Nicoletti et al., 1998; Chen et al., 2005; Lim and Zhu, 2006; Massague, 2008; Town et al., 2008; Heinemann et al., 2012). However, there are quite numbers of studies reported in the activation of TGF-β in TBI (Lindholm et al., 1992; Morganti-Kossmann et al., 1999; Zhang et al., 2013), the downstream mechanisms of TGF-β in inflammatory and apoptotic cascades in the context of mTBI is not well studied. The purpose of this study is to elucidate the mechanisms by which TGF-β1 triggers neuroinflammation and neurodegeneration using an *in vitro* neuronal stretch injury model and validate the pathway in an animal model of fluid percussion injury (FPI) (Abdul-Muneer et al., 2016, 2017). We test the hypothesis that TBI-TGF-β1 initiates neuroinflammation and neurodegeneration via the phosphorylation of Smad2 and Smad3 proteins. Hence, targeting NOX and TGF-β1 with pharmacological/molecular levels presents new therapeutic possibilities for the treatment of TBI-related neurological diseases.

2. Materials and methods

Reagents: The primary antibodies mouse anti-NeuN (Cat. No. ab104224), rabbit anti-4HNE (Cat. No. ab46545), anti-IL-1-β (Cat. No. ab2105), anti-GFAP (Cat No. ab4648), anti-Iba1 (Cat No. ab5076), and anti-cleaved PARP p85 (Cat. No. ab32064) were purchased from Abcam (Cambridge, MA). Rabbit anti-TNF-α (Cat. No. 11948S), anti-Smad2 (Cat. No. 5339S), anti-p-Smad2 (Cat. No. 3108S), anti-Smad3 (Cat. No. 9513S), and anti-p-Smad3 (Cat. No. 9520S) were purchased from Cell Signaling (Danvers, MA). Rabbit anti-NOX1 (Cat. No. SAB4200097) and anti-TGF-β1 (Cat. No. AV37894) were from Sigma-Aldrich (St. Louis, MO); mouse anti-β-actin (Cat. No. PIMA515739) and rabbit anti-annexin V (Cat. No. PA5-27872) were from Thermo scientific (Rockford, IL); and rabbit anti-cleaved caspase-3 (Cat. No. MAB835) was from R and D systems (Minneapolis, MN). All secondary Alexa Fluor conjugated antibodies, TUNEL kit, and DAPI were purchased from Invitrogen (Carlsbad, CA, USA); apocynin (inhibitor of NADPH oxidase), and SB431542 (TGF-β type I receptor) were purchased from Cayman chemicals (Ann Arbor, MI), FluoroJade C (Cat No. AG325) was purchased from Sigma Aldrich (St Louis, MO), and TGF-β1 siRNA was purchased from Santa Cruz Biotechnology (Dallas, TX).

2.1. Neuronal culture

Cortical neurons were isolated from E17 Sprague-Dawley rat embryos (Abdul-Muneer et al., 2016, 2017). Briefly, after rinsing with Ca^{2+} and Mg^{2+} free HBSS, the rat brain cortices were digested with 0.25% trypsin containing EDTA (0.2 g/L) (Sigma-Aldrich, Cat. No. T4049) and DNase I (1.5 mg/mL) at 37 °C for 30 min, neutralized with 10% fetal bovine serum, and further dissociated by trituration. The dissociated cells were filtered with 100 μm and 40-μm pore sized cell strainers (EMD Millipore, Billerica, MA) to remove large pieces of tissue. Neurons were cultured (300,000 cells/well) on a poly-D-lysine (0.1 mg/mL) coated elastic silicone membrane mounted on Bioflex culture plates (Flexcell International Corp, Burlington, NC) in NeuroBasal media containing 2% B-27, 1% penicillin-streptomycin and 0.4 mM L-glutamine at 37 °C in a CO_2 (5%) incubator. Cultured cells were fed every two days. The purity of neurons was assessed by immunostaining with anti-NeuN antibody (Abdul-Muneer et al., 2016), which normally showed 100% enrichment of neurons.

2.2. Neuronal stretch injury

On day 10 of neuronal culture, the cells were subjected to stretch injury (biaxial) with a pressure of 2.0 psi using an *in vitro* cell injury device called 'cell injury controller II' (Custom design and fabrication Inc., Sandstom, VA) (Geddes-Klein et al., 2006; Salvador et al., 2013; Abdul-Muneer et al., 2016, 2017). For experiments with pharmacological treatments, neurons were treated with SB431542 or apocynin 30 min prior to the stretch injury. 24 h post stretch injury, cell culture supernatant was collected for cytokine analysis and cells were fixed for immunostaining or TUNEL and proteins were extracted for western blotting.

2.3. TGF-β1 gene knockdown

TGF-β1 siRNA transfection was conducted as per the manufacturer's instruction (Santa Cruz Biotech, Dallas, TX). The cells were incubated at 37 °C in a CO_2 incubator until the cells were 60–80% confluent (approximately 18–24 h). Briefly, E17 rat neurons cultured on injury wells were washed once and incubated with 1 mL siRNA transfection medium containing 2–8 μl siRNA duplex (0.25–1 μg siRNA) for 6 h at 37 °C in a CO_2 incubator. Then 1 ml of normal growth medium containing 2 times the normal serum and antibiotic concentration (2x normal growth medium) was added without removing the transfection mixture and the cells were incubated for an additional 18–24 h. The medium was replaced with fresh 1X normal growth medium and the stretch injury was conducted. The scrambled control siRNA was used to compare the effect of TGF-β1 siRNA. 24 hrs post stretch injury, cells were fixed for immunostaining or proteins were extracted for western blotting.

2.4. Smad7 gene transfection

Smad7 is an inhibitor of TGF-β signaling. It prevents TGF-β-dependent formation of Smad2/Smad4 complexes and inhibits the nuclear accumulation of Smad2 (Hayashi et al., 1997). Plasmids carrying Smad7 (pCMV5-Smad7-HA) was obtained from Addgene