

Role of Smad3 inhibitor and the pyroptosis pathway in spinal cord injury

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Abstract. The aim of the present study was to investigate the role of Smad3 inhibitors and the pyroptosis pathway in spinal cord injury, and to determine the underlying mechanism. The pyroptosis signaling pathway may be involved in spinal cord injury during the recovery period. Smad3 inhibitor may serve a role in alleviating spinal cord injury by reducing the pyroptosis of neurons, which is induced by caspase-1, absent in melanoma-2 or NOD-like receptors protein-1 during the recovery period of spinal cord injury. In the present study, spinal cord injury was alleviated by caspase-1 and Smad3 inhibitors. Therefore, a Smad3 inhibitor could relieve spinal cord injury in mice by directly downregulating caspase-1 and reducing neuron pyroptosis following spinal cord injury during the recovery period.

Introduction

Spinal cord injury (SCI) is a serious condition with high mortality and poor prognosis following spinal trauma, including primary and secondary injury. Primary injury often occurs during the first 48 h after injury while the secondary injury, which occurs a few days to several months after SCI, is more destructive and considered as a determining factor of the repair of damaged neurons (1). The tumor growth factor β (TGF- β)/Smad pathway serves an important role in secondary SCI (2). Growth differentiation factor-11 (GDF-11), a member of the TGF- β superfamily, is recognized by activin type II receptor A and -B, and phosphorylates Smad2 and -3. Following phosphorylation, Smad2/3 binds to Smad4 to form a

complex, which regulates gene expression and accumulates in the nucleus. A previous study suggested that the GDF-11/Smads pathway was associated with cell growth, differentiation and homeostasis (3). Notably, Shi and Liu (3) demonstrated a slower neuronal differentiation rate in GDF-11^{-/-} mouse embryos during spinal cord development. GDF-11 signals produced by newly differentiated neurons act as a feedback signal on the adjacent progenitors to promote cell cycle exit, inhibit proliferation, and thus facilitate temporal progression of neurogenesis in the developing spinal cord (4). This indicates that GDF-11 may play a role in neurogenesis in the spinal cord.

Pyroptosis is a caspase-1-dependent, inflammatory form of programmed cell death (5). Several previous studies have only focused on the role of pyroptosis in macrophages and dendritic cells (6-9). However, a recent study demonstrated that the pyroptosis pathway may also play a role in nerve cells (10). Pyroptosis-associated proteins, including apoptosis-associated speck-like protein containing a caspase-activating recruitment domain (ASC), caspase-1 and interleukin (IL)-1 β , may play a role in the innate immune response against bacterial infection or trauma. The inflammasome is formed after injury of the central nervous system via absent in melanoma-2 (AIM-2) (11) or NOD-like receptors protein (NLRP)-1 (12), which induce cleavage of pro-caspase-1. Activated caspase-1 serves a key role in pyroptosis through the induction of IL-1 β and IL-18, which are involved in the regulation of inflammation (13).

Therefore, the pyroptosis pathway may be involved in the pathological process of SCI and may be a therapeutic target for alleviating secondary injury. The aim of the present study was to investigate the roles of the GDF-11/Smad and pyroptosis pathways in SCI during the second injury period, and to determine the underlying mechanism.

Materials and methods

Animals and design. The present study used ICR wild-type mice (6-8 week old; male; n=120 in total) purchased from The Animals Center of China Medical University. The mice were housed in a 12-h light/dark cycle at 26°C with 50% humidity and free access to food and water, except where otherwise indicated. SCI was established by artery clamp through

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clamping of the T10 level spinal cord after laminectomy for 1 min according to a previous study by Marques *et al* (14). Control mice received a sham-operation, which included a laminectomy without SCI. Ethical standards of China Medical University were followed, and the present study was approved by the local Animal Committee of China Medical University.

ICR wild-type mice were randomly divided into six groups (n=10 per group). The mice of the normal saline (N) + control (con) group were treated with 20 μ l normal saline at T10 level through a local intraspinal epidural injection after sham-operation. After SCI was established, the mice of the N+sci group were treated with 20 μ l normal saline at the injured level through local injection. The mice of the caspase-1 (C) + con group were treated with caspase-1 inhibitor (cat. no. sc-358878; Santa Cruz Biotechnology, Inc.; 20 μ g in 20 μ l normal saline) at the T10 level of the spinal cord after sham-operation. The SCI mice in the C+sci group were also injected with caspase-1 inhibitor at the T10 level. The mice of the Smad3 inhibitor (S) + con group were treated with Smad3 inhibitor (cat. no. sc-222318; Santa Cruz Biotechnology, Inc.; 20 μ g in 20 μ l normal saline) at the T10 level through local injection, and the mice in the S+sci group were treated with Smad3 inhibitor at the injured level after SCI. Basso Mouse Scale (BMS) scores (15) were used to assess the recovery of the injured mice during the first 2 weeks after operation. The behaviors of the mice were observed and the degree of SCI was assessed by BMS scores prior to sacrifice. Behavioral changes, including significant decrease of body temperature, respiratory depression and bradycardia in SCI mice were considered as humane endpoints where the mice would be sacrificed by the staff of The Animal Department of China Medical University. The mice were sacrificed and the injured level of the spinal cord was harvested on the 14th day postoperatively, except where otherwise indicated. All animal procedures were performed to minimize suffering in accordance with the guidelines established by The Animal Experimental Committee.

Western blot analysis. The spinal cord tissues (a length of 6 mm including the injured tissue) were homogenized in Laemmli buffer (cat. no. 1610737; Bio-Rad Laboratories, Inc.), and the proteins (50 μ g per lane determined using a Bradford Protein Assay kit; cat. no. P0006; Beyotime Institute of Biotechnology) were separated by 10% SDS-PAGE, then transferred to PVDF membranes. The membranes were blocked with 1% BSA at room temperature for 1 h and incubated sequentially with primary antibodies (1:1,000 dilution; room temperature; 2 h) and secondary antibodies (1:2,000 dilution; room temperature; 1 h). β -actin was used as the internal control. Western blotting was performed using antibodies against caspase-1 (cat. no. sc-56036; Santa Cruz Biotechnology, Inc.), IL-1 β (cat. no. 12703; Cell Signaling Technology, Inc.), GDF-11 (cat. no. sc-81952; Santa Cruz Biotechnology, Inc.), Smad4 (cat. no. sc-7966; Santa Cruz Biotechnology, Inc.), NLRP1 (cat. no. QC49289; Sigma Aldrich; Merck KGaA), AIM-2 (cat. no. ab180655; Abcam), ASC (cat. no. sc-22514-R; Santa Cruz Biotechnology, Inc.) and β -actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). HRP-conjugated goat anti rabbit IgG (cat. no. ZB-2301; OriGene Technologies, Inc.) or HRP-conjugated goat anti mouse IgG (cat. no. ZB-2305;

OriGene Technologies, Inc.) were used as secondary antibodies. ECL Western Blotting Substrate (cat. no. 32106; Thermo Fisher Scientific, Inc.) was used as the visualization reagent (Image Lab V5.2.1; Bio-Rad Laboratories, Inc.).

Immunohistochemistry and immunofluorescence. Spinal cords were fixed overnight with 4% formaldehyde in PBS (pH 7.2) at room temperature, carefully isolated, embedded in paraffin and cut into 5- μ m sections. The sections were first incubated with 3% BSA at room temperature for 1 h to prevent endogenous interference. The sections were then incubated with primary antibodies (1:100 dilution) at room temperature for 2 h, followed by secondary antibodies (1:200 dilution) labeled with fluorescence or horseradish peroxidase (room temperature; 1 h). The following primary antibodies were used for analysis: Caspase-1 (cat. no. sc-56036; Santa Cruz Biotechnology, Inc.), GDF-11 (cat. no. sc-81952; Santa Cruz Biotechnology, Inc.), Smad2/3 (cat. no. 5678; Cell Signaling Technology, Inc.), NLRP1 (cat. no. QC49289; Sigma-Aldrich; Merck KGaA) and AIM-2 (cat. no. ab180655; Abcam). The following secondary antibodies were used for analysis: HRP-conjugated goat anti rabbit IgG (cat. no. ZB-2301; OriGene Technologies, Inc.), HRP-conjugated goat anti mouse IgG (cat. no. ZB-2305; OriGene Technologies, Inc.), Cy3-conjugated goat anti-mouse IgG (cat. no. ab97035; Abcam) and FITC-conjugated goat anti-rabbit IgG (cat. no. ab6717; Abcam). Images were acquired using fluorescence microscopy (magnification, x400). Representative images were obtained from at least two different sections.

Statistical analysis. The data are presented as the mean \pm standard deviation. Data were analyzed using Student's t-test, except for multiple comparisons, where ANOVA was used instead. Tukey's post hoc test was used following ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference. All analyses were performed using SPSS software (V16.0; SPSS, Inc.) and all experiments were repeated three times.

Results

Pyroptosis signaling pathway may be involved in SCI during the recovery period. No mice died in the con group and the survival rate of this group was 100% over 2 weeks. After the sham operation, hind limb dysfunction of this group was not observed and the BMS scores were 9. In the SCI group, the survival rate decreased to 77.78% at day 3 following surgery (Fig. 1A), and a slight ankle movement was observed in 7 of the 9 mice in this group. The mean BMS score was 0.64 ± 0.44 . On day 5 after surgery, significant ankle joint activity was observed in the majority of mice in the SCI group, while a large range of active ankle joint activity and occasionally landing on the instep was observed in 3 of the 9 mice. The mean BMS score was increased to 1.36 ± 1.05 and the survival rate of the SCI group was reduced to 55.56% compared with the con group. Limb dysfunction observed in mice from the SCI group gradually stabilized between days 7 and 14 after SCI, and the mean BMS score increased to 3.89 ± 1.76 . The BMS scores were significantly different between the SCI and con groups between days 3 and 14 (Fig. 1B).

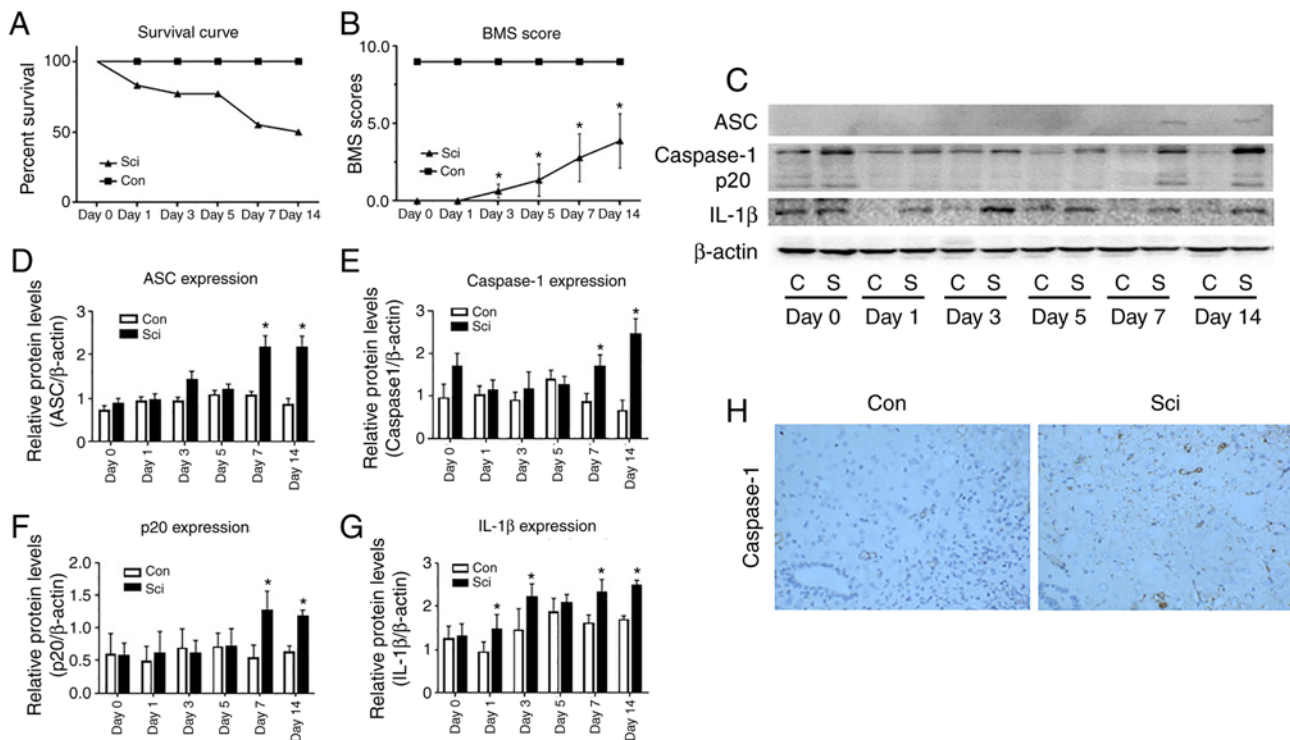


Figure 1. Pyroptosis signaling pathway may be involved in SCI during the recovery period. In the sci group, SCI injury was established by artery clamp through clamping of the T10 level spinal cord for a minute after laminectomy. In the con group, mice received a sham operation. (A) Survival curve of SCI mice 14 days after operation. (B) BMS score of Sci mice. (C) Expression of pyroptosis pathway markers. Mice were sacrificed after the operation and the spinal cords (6 mm including the injured level) were obtained for western blot analysis. The molecular weight of caspase-1 is ~51 kDa and mature form caspase-1 (p20) is ~20 kDa. (D) Statistical analysis of ASC western blot results. (E) Statistical analysis of caspase-1 western blot results. (F) Statistical analysis of p20 western blot results. (G) Statistical analysis of IL-1 β western blot results. (H) Injured spinal cord tissue was harvested for immunohistochemistry at day 14 after surgery. Magnification, x400. * $P < 0.05$ vs. respective con group. SCI/sci/S, spinal cord injury; Con/C, control; ASC, apoptosis-associated speck-like protein containing a caspase-activating recruitment domain; IL, interleukin; BMS, Basso Mouse Scale.

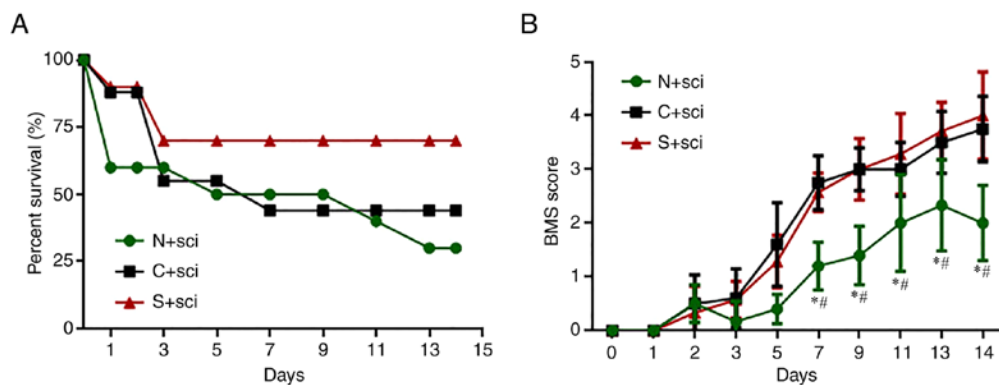


Figure 2. Survival rate and BMS scores of SCI mice during the first 2 weeks. SCI mice were treated with 20 μ l normal saline (N+sci) caspase-1 inhibitor (C+sci) or Smad3 inhibitor (S+sci) during operation. (A) Survival curve 14 days after operation. (B) BMS scores of sci mice. * $P < 0.05$ vs. S+sci group; # $P < 0.05$ vs. C+sci group. SCI/sci, spinal cord injury; C, caspase-1 inhibitor; S, Smad3 inhibitor; N, normal saline; BMS, Basso Mouse Scale.

ASC and caspase-1 were highly expressed in the spinal cords of mice in the SCI group at days 7 and 14 (Fig. 1D and E). By contrast, ASC and caspase-1 were hardly expressed in the spinal cord of the con group (Fig. 1C). In addition, the caspase-1 p20 subunit was detected in the SCI group (Fig. 1F), but not in the con group at days 7 and 14. The expression of IL-1 β was significantly increased in the SCI group, compared with the con group at days 1, 3, 7 and 14 ($P < 0.05$; Fig. 1C and G).

The expression of caspase-1 at the T10 spinal cord level was detected by immunohistochemical staining at day 14 after

surgery. It was identified that caspase-1 was highly expressed and predominantly distributed in the anterior horn of the spinal cord in SCI group mice, while it was rarely expressed in the con group (Fig. 1H).

SCI is alleviated by caspase-1 inhibitors and Smad3 inhibitors. At the first 48 h after SCI, the survival rate was reduced markedly to 60% in the N+sci group, 90% in the S+sci group and the 88% in C+sci group (Fig. 2A). There were no obvious differences in the survival rates of the C+sci and N+sci groups

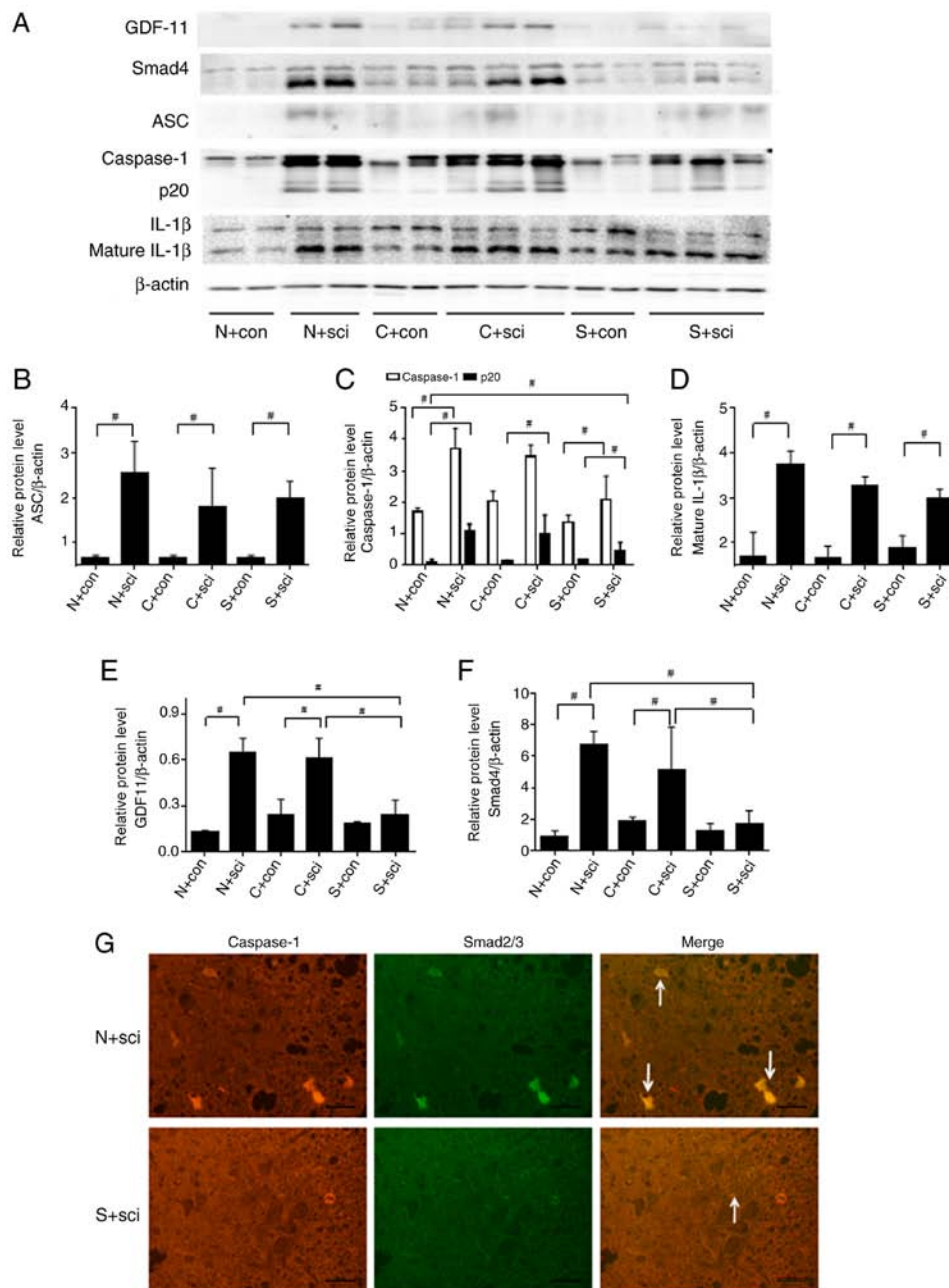


Figure 3. Association between the GDF-11/Smads pathway and the pyroptosis pathway in SCI during the recovery period. Mice were sacrificed on day 14 after operation, and injured tissue from spinal cords was harvested for western blot analysis and immunofluorescence. (A) Expressions of pyroptosis pathway markers and GDF-11/Smad4 were detected by western blotting. (B) Statistical analysis of ASC expression. (C) Statistical analysis of caspase-1 expression. (D) Statistical analysis of IL-1 β expression. (E) Statistical analysis of GDF-11 expression. (F) Statistical analysis of Smad4 expression. (G) Immunofluorescence co-staining of caspase-1 and Smad2/3 in spinal cord. Magnification, $\times 400$. Scale bars represent 50 μm . Positive expression is indicated by the white arrows in merged images. * $P < 0.05$. GDF-11, growth differentiation factor-11; SCI/sci; spinal cord injury; Con, control; C, caspase-1 inhibitor; S, Smad3 inhibitor; N, normal saline; ASC, apoptosis-associated speck protein containing a caspase-activating recruitment domain; IL, interleukin.

between days 4 and 14. However, the survival rate of the S+sci group was markedly higher than the other two groups (Fig. 2A).

The BMS score was 0 for all three SCI groups at the first day after operation. The scores then gradually increased during the first 5 days and there were no significant differences among the three groups ($P > 0.05$). The BMS scores of the S+sci and C+sci groups were markedly increased between days 7 and 14, compared with the N+sci group (Fig. 2B). There were no significant differences in the BMS scores between the C+sci and S+sci groups.

Smad3 inhibitors inhibit caspase-1-induced pyroptosis of neurons in SCI mice. Markers of the pyroptosis signaling pathway, including ASC, p20 and mature IL-1 β , were expressed at low levels in the three control groups. No significant difference was identified in the expression of ASC between the three SCI groups (Fig. 3B). Mature IL-1 β in SCI mice was downregulated following treatment with caspase-1 inhibitor compared with the N+sci group (Fig. 3A). The expression levels of p20 and IL-1 β were significantly decreased in the S+sci group, compared with the N+sci group ($P < 0.05$; Fig. 3C-D). The caspase-1 fragment p20 was detectable following SCI in the

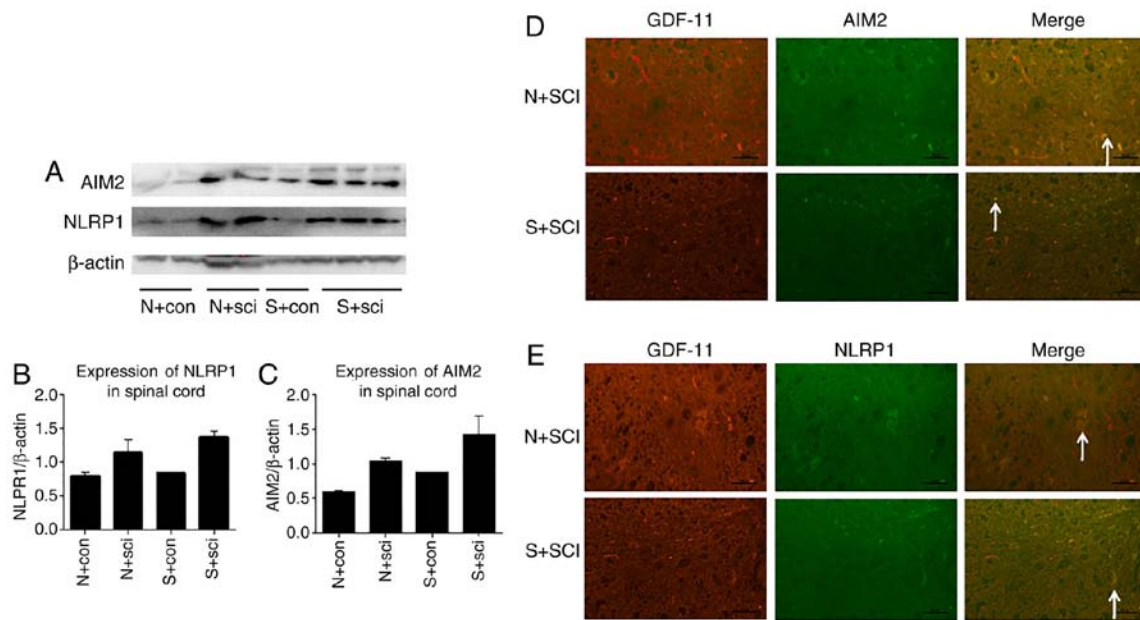


Figure 4. AIM2 and NLRP1 expression in spinal cord. Mice were sacrificed on day 14 after the operation and the spinal cord was harvested for western blot analysis or immunofluorescence co-staining. (A) AIM2 and NLRP1 were detected by western blotting. (B) Statistical analysis of NLRP1 western blots. (C) Statistical analysis of AIM2 western blots. (D) Immunofluorescence co-staining of GDF-11 and AIM-2. (E) Immunofluorescence co-staining of GDF-11 and NLRP1. Magnification, $\times 400$. Scale bars represent $50\ \mu\text{m}$. Positive expression is indicated by the white arrows in merged images. AIM-2, absent in melanoma-2; NLRP1, NOD-like receptor protein-1; GDF-11, growth differentiation factor-11; S, Smad3 inhibitor; N, normal saline; SCI/sci, spinalcord injury; con, control.

N+sci group, and downregulated in the S+sci group compared with the other two SCI groups (Fig. 3A and C).

The expression levels of GDF-11 and Smad4 were increased after SCI in the N+sci group and expressed at low levels in the S+sci group, compared with the other two SCI groups at day 14 after injury (Fig. 3A). No significant differences were identified in the expression levels of GDF-11 and Smad4 between the C+sci and N+sci groups (Fig. 3A, E and F).

Co-immunostaining demonstrated that Smad2/3 expression was enriched in caspase-1-expressing neurons at the ventricornu of the N+sci group. The expression of Smad2/3 and caspase-1 could be inhibited and the number of pyroptotic neurons was decreased by treating the SCI mice with the Smad3 inhibitor (Fig. 3G).

AIM-2 or NLRP1 may not be targets of the GDF-11/Smads pathway in the regulation of pyroptosis in neurons. The expression levels of NLRP1 and AIM-2 were markedly increased in the N+sci and S+sci groups, compared with the respective controls, while there were no significant differences in NLRP1 and AIM-2 expression between these two groups (Fig. 4). Co-immunostaining of GDF-11 with AIM-2 or NLRP1 in ventricornu demonstrated no association between GDF-11 and AIM-2 or NLRP1 (Fig. 4D and E).

Discussion

Pyroptosis is a distinct form of programmed cell death triggered by the activation of the ASC/caspase-1 pathway. Unlike apoptosis, cells undergoing pyroptosis enhance the release of the pro-inflammatory factors IL-1 β and IL-18 (16,17). Previous studies demonstrated that the pyroptosis pathway mediates host defense and induces a secondary immunological response

to infectious diseases or damage (18,19). Pro-caspase-1, a large protein of ~ 51 kDa, is activated by ASC and cleaves into the activated form p20 (~ 20 kDa) to induce pyroptosis. de Rivero Vaccari *et al* (19) suggested that ASC expression increased and caspase-1 was activated in rats following SCI, compared with control rats during primary injury. It was suggested that neuronal pyroptosis may serve an important role in SCI. The present study demonstrated that pyroptosis of spinal cord neurons may be involved in SCI during the recovery period. Thus, inhibition of neuronal pyroptosis could alleviate secondary SCI and may be an underlying therapeutic target for SCI.

In the present study, the highest mortality in the N+sci group was observed 24 to 72 h after SCI, which could be attributed to secondary injury of the spinal cord, including nerve cell death, the release of inflammatory cytokines, calcium overload and peroxidation which often gradually occurs at 24 to 72 h after injury (20-22). In a previous study by Li *et al* (23), the caspase inhibitor zVAD-fmk ($10\ \mu\text{g}$ in $10\ \mu\text{l}$ vehicle) could block the caspase-1 pathway in C57BL/6 mice (20-25 g) following SCI.

To understand the role of the pyroptosis signaling pathway in SCI, the present study focused on ICR mice (30-35 g) treated with caspase-1 inhibitor according to the previous study by Li *et al* (23). Following SCI, the survival rate of mice treated with the caspase-1 inhibitor was markedly improved during the first 2 days, compared with the control group. The BMS scores were also markedly higher with caspase-1 inhibition, compared with the control. This suggested that SCI could be alleviated by inhibiting the pyroptosis signaling pathway via caspase-1. Inhibition of the pyroptosis pathway might reduce the injury of nerve cells during secondary injury, decrease the release of inflammatory cytokines, including IL-1 β , and suppress neuronal pyroptosis.

The member of the TGF- β superfamily GDF-11 is involved in cell growth and differentiation (4). A previous study demonstrated that GDF-11 was associated with neurogenesis, and spinal cord neural progenitor cells that do not express GDF-11 fail to exit the cell cycle, resulting in alteration of neural subtypes (3). GDF-11 exerts its function by interacting with its receptors to induce phosphorylation and activation of Smad2 and Smad3. Smad2/3 assembles with Smad4 to form complexes in the nucleus that regulate target genes (24-26). The present study demonstrated that the expression levels of GDF-11 and Smad4 increased significantly in N+sci and C+sci group, compared with the respective control groups, indicating that the GDF-11/Smads pathway is activated following SCI. To understand the role of the GDF-11/Smads signaling pathway in SCI, a Smad3 inhibitor was used in the early injury period to inhibit this pathway. In the present study, the survival rate of mice treated with Smad3 inhibitor was markedly improved and BMS scores were higher from days 7 to 14 after spinal cord injured, compared with that of the N+sci group. Therefore, SCI could be alleviated by blocking the GDF-11/Smads pathway. Compared with the control group, the expression levels of p20 and IL-1 β in the Smad3-inhibitor-treated group mice significantly decreased, which suggested that the pyroptosis signaling pathway in SCI mice could be suppressed by a Smad3 inhibitor. There may be an underlying association between the GDF-11/Smads and pyroptosis pathways in SCI during the recovery period. Therefore, Smad3 inhibitors may serve a role in alleviating SCI in mice by reducing neuronal pyroptosis induced by caspase-1.

Tamai R and Kiyoura (27) demonstrated that alendronate could augment lipid A-induced IL-1 β release and Smad3/NLRP-3/ASC-dependent J774.1 cell death, and that Smad3 inhibition could suppress alendronate-augmented caspase-1 and IL-1 β production. These previous results were consistent with the findings of the present study. While the previous study focused on macrophage pyroptosis and investigated the role of NLRP-3, a marker of the macrophage-mediated inflammation, the present study examined pyroptosis in nerve cells.

AIM2 and NLRP1 are components of inflammasomes in neurons, which contribute to the activation of pro-caspase-1 and the induction of pyroptosis (11,12). To understand the regulation of the Smad3 inhibitor in the pyroptosis pathway of neurons, NLRP1 and AIM-2 expression in SCI mice was assessed in the present study. There were no differences in the NLRP1 and AIM-2 expression levels between the Smad3 inhibitor and the control groups, which suggested that AIM-2 and NLRP1 may not be the targets of the GDF-11/Smads pathway that serve a role in the regulation of neural cell pyroptosis. The expression levels of caspase-1 and Smad2/3 in the spinal cord were detected by immunofluorescence co-staining. Smad2/3 and caspase-1 were strongly co-expressed in neurons undergoing pyroptosis in the control group. The expressions of Smad2/3 and caspase-1, as well as the extent of pyroptosis, were decreased by treating SCI mice with the Smad3 inhibitor. This suggested that Smad3 may directly regulate the expression of caspase-1 and affect pyroptosis.

In summary, the present study demonstrated that inhibition of the GDF-11/Smads pathway could relieve SCI by directly downregulating caspase-1 to reduce pyroptosis of

neurons during the recovery period after SCI. The underlying mechanism of this remains unclear and requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ carried out the experiment, participated in the analysis and interpretation of data and drafted the manuscript. YF participated in the analysis of the data and performed the statistical analysis. GT conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the local Animal Committee of China Medical University. Ethical standards of China Medical University were followed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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