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ORIGINAL ARTICLE

Effect of adenovirus-mediated RNA interference of IL-1β expression on spinal cord injury in rats

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Study design: We introduced an adenoviral vector expressing interleukin-1β (IL-1β) small-hairpin RNA (shRNA) into the injured spinal cords to evaluate the therapeutic potential of IL-1β downregulation in a rat model of spinal cord injury (SCI).
Objectives: The purpose of this study was to investigate the possible protective effects of the IL-1β downregulation on traumatic SCI in rats.
Setting: Department of Orthopedic Surgery, The Second Affiliated Hospital, Fujian Medical University, Quanzhou, People's Republic of China.
Methods: An adenoviral shRNA targeting IL-1β was constructed and injected at the T12 section 7 days before SCI. The rats' motor functions were evaluated by the Basso–Beattie–Bresnahan (BBB) rating scale. Immunofluorescence, enzyme-linked immunosorbent assay, flow-cytometric analysis and western blots were also performed.
Results: Animals downregulating IL-1β had significantly better recovery of locomotor function and less neuronal loss after SCI. In addition, IL-1β downregulation significantly decreased tumor necrosis factor-alpha (TNF-α) level and Bax expression, reduced the activity of caspase-3 and increased Bcl-2 expression after SCI.
Conclusion: This study demonstrated that the IL-1β downregulation may have potential therapeutic benefits for both reducing secondary damages and improving the outcomes after traumatic SCI.
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INTRODUCTION

Traumatic spinal cord injury (SCI) includes primary and secondary injury mechanisms. Primary injury is caused by the mechanical impact leading to direct contusion, shearing injury or laceration of the spinal cord. After the primary injury, the spinal cord will undergo a few sequential pathologic changes including ischemia, free radical release, lipid peroxidation, inflammation and apoptosis.[1] In contrast to the primary and irreversible trauma, the secondary effects can potentially be influenced, thereby offering possibilities for therapeutic approaches. Although some therapeutic agents are being used to protect the injured spinal cords from those secondary pathological processes,[2] there is still no effective treatment for SCI.

Interleukin-1β (IL-1β) has a crucial role in SCI. It was demonstrated that IL-1β is a key inflammatory mediator that is increasingly expressed after SCI,[3–5] and the inflammatory process may impede neuronal repair leading to secondary spinal cord damage.[6] Furthermore, increased IL-1β in the spinal cord has been associated with neuronal apoptosis.[7,8] In addition, injection of an IL-1 receptor antagonist (IL-1Ra) reduces IL-1β production in the spinal cord and thereby promotes functional recovery following SCI.[9]

RNA interference is a method of blocking gene function by inserting short sequences of RNA that match part of the target gene's sequence.[10,11] There are two RNA interference approaches: small-interfering RNA and small-hairpin RNA (shRNA).[12] ShRNA has proved to be an effective and a long-term silencing mechanism.[13] It allows stable suppression of functions not only in cell culture but also in animals.[14,15] In the current study, we used adenovirus-mediated shRNA technology to inhibit the expression of IL-1β in spinal cord and then observed the therapeutic effect of rats with SCI.

MATERIALS AND METHODS

Production of the Ad-shIL-1β

To produce adenoviral shRNA targeting IL-1β (Ad-shIL-1β), shRNA sequence containing small-interfering RNA target 5′-ATGAACAACAAAAATGCCTC G-3′ was synthesized and inserted into pDC311-U6-MCMV-EGFP vector (purchased from Hanbio Co. Ltd, Shanghai, China). The pDC311-IL-1β-shRNA and pBHGlox E1, 3Cre[16,17] were co-transfected into HEK293 cells by using LipoFiter transfection reagent (Hanbio) to generate the recombinant adenoviruses (Ad-shIL-1β). Adenoviruses harboring green fluorescent protein (Ad-GFP) were used as a control. Ad-shIL-1β and Ad-GFP were propagated in HEK293 cells. The propagated recombinant adenoviruses in the HEK293 cells were purified, and the titer of virus was measured by plaque assays. Titer of Ad-shIL-1β was 1.2×10^{11} plaque formation unit per ml and that of Ad-GFP was 1.1×10^{11} plaque formation unit per ml.

The SCI model and experimental groups

Seventy-two adult male Sprague-Dawley rats (280–320 g) were used in this study. The animal experiments were performed in accordance with the

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guidelines of Laboratory Animals and were approved by the Animal Care and Use Committee of Fujian Medical University.

Spinal cord compression at T12 was performed following a previously established static compression model, as described in our previous paper.[18] Briefly, animals were anesthetized by inhalation of 2–3% isoflurane administered at a flow rate of 1 l min^{-1}. Midline skin incisions were performed to expose the T12 spinous processes. A laminectomy was performed at T12. The compression was applied by suspending the base of a compression platform (area $2 \times 5\ mm^2$) onto the exposed cord. A weight of 50 g was applied statically to the platform for exactly 5 min. After removing the platform, the muscles and skins were sutured.

The rats were randomly assigned into four groups ($n = 18$ per group) including the Sham, the Vehicle, the Ad-GFP and the Ad-shIL-1β groups. In the Sham group, a laminectomy was performed only. In the Vehicle group, 3 μl normal saline was injected into the cord at T12 using a 5-μl micro-syringe with a 33-gauge needle (Hamilton, Reno, NV, USA) at a rate of 0.2 μl min^{-1},[19,20] 7 days before SCI. In the Ad-GFP group, 3 μl Ad-GFP was injected 7 days before SCI. In the Ad-shIL-1β group, 3 μl Ad-shIL-1β was injected 7 days before SCI. After removal of the injectors, muscles and skins were sutured in separate layers.

Neurologic evaluation

The hindlimb locomotor function was assessed at pre-injury and 1, 3, 7, 14 and 21 days after SCI using the Basso–Beattie–Bresnahan (BBB) locomotor test developed by Basso *et al.*[21] The hindlimb movements during locomotion were quantified using a scale ranging from 0 to 21. The rats were observed for 5 min at each time point by two observers who were blinded to the experimental protocol.

Immunochemical staining

The spinal cord sections from the compression epicenter were incubated with antibodies against NeuN (1:100; Invitrogen Life Technologies, Carlsbad, CA, USA) overnight at 4 °C. The sections were then incubated with Alexa Fluor 488-conjugated goat anti-rat IgG (Invitrogen Life Technologies). Fluorescent microscopy was conducted using an Axio Observer Z1 fluorescent microscope (Zeiss, Baden-Württemberg, Germany). Six sections were randomly selected from each animal, and six microscopy fields of each section were randomly selected for quantification of NeuN+ cells. A total of six rats were analyzed in each group. The positive staining in each section was measured using the Image J software (National Institutes of Health, Bethesda, MD, USA).

Measurement of tumor necrosis factor-alpha levels

Spinal cord segments with lesions were harvested at 24 h after SCI. Spinal cords were lysed and homogenized. The levels of tumor necrosis factor-alpha (TNF-α) were analyzed in the supernatants of tissue homogenates using enzyme-linked immunosorbent assay kits (Abcam, Cambridge, MA, USA) according to the manufacturer's guidelines.

Isolation of leukocytes from spinal cord and flow-cytometric analysis

Spinal cord was harvested and then mechanically dissociated with the edge of a syringe. Cells were suspended in phosphate-buffered saline (PBS) buffer and then passed through a 70-μm nylon cell strainer (Becton Dickinson, San Jose, CA, USA) to isolate tissue debris. The cell suspension was centrifuged at 300 *g*, 4 °C for 5 min. The cell pellet was re-suspended in PBS and then lysed in 1× RBC lysis buffer. Cells were then washed with PBS and incubated with the following anti-rat antibodies on ice for 15 min: FITC anti-Gr-1 (RP-1, BD Bioscience, San Jose, CA, USA), PE/Cy7 anti-CD45 (OX-1, Biolegend, San Diego, CA, USA), PE anti-CD11b/c (OX-42, Biolegend) and FITC anti-CD3 (eBioG4.18, eBioscience, San Diego, CA, USA). Samples were washed with PBS and loaded on a BD Calibur flow cytometer (BD Biosciences). To avoid debris, prepared spinal cord cells were stained with 7-Amino-Acinomycin D (BD Bioscience), which is a dye for dead cells. The data were analyzed using the FlowJo software (BD Bioscience).

Western blot analysis

The protein homogenates of the spinal cord samples were prepared by rapid homogenization in lysis buffer, and the lysates were separated by 10% SDS–PAGE. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After incubation with rabbit anti-IL-1β (1:400; Santa Cruz Biotechnology, Paso Robles, CA, USA), rabbit anti-Bax (1:500; Abcam), rabbit anti-Bcl-2 (1:500; Abcam), rabbit anti-cleaved caspase-3 (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-GFAP (1:400; Santa Cruz Biotechnology) or rabbit anti-β-actin (1:400; Cell Signaling Technology), the membrane was incubated with HRP-conjugated secondary antibody in TBS-T for 1 h at room temperature. Immunoreactive complexes were visualized by ECL and exposed to X-ray film. The protein signals were quantified by scanning densitometry using AlphaEaseFC (AlphaInnotech, San Leandro, CA, USA). The results from each experimental group were expressed as the relative integrated intensity compared with β-actin.

Statistical analysis

All data were presented as the mean ± s.d. One-way analysis of variance (ANOVA) was used to compare the IL-1β, TNF-α, Bax, Bcl-2, cleaved caspase-3 and glial fibrillary acidic protein (GFAP) levels and neuron density among the groups. The BBB scale data were analyzed with repeated-measures ANOVA. A P-value of less than 0.05 was considered as significant. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Ad-shIL-1β decreases IL-1β expression in the spinal cord

As shown in Figure 1, the levels of the IL-1β protein expression were determined by western blot analysis. The levels of IL-1β were low in the spinal cord of the Sham group, which increased 24 h after SCI. Compared with the Sham group, the levels of IL-1β were significantly higher in the spinal cord of the Vehicle, the Ad-GFP and the Ad-shIL-1β groups ($P < 0.05$). However, the levels of the IL-1β in

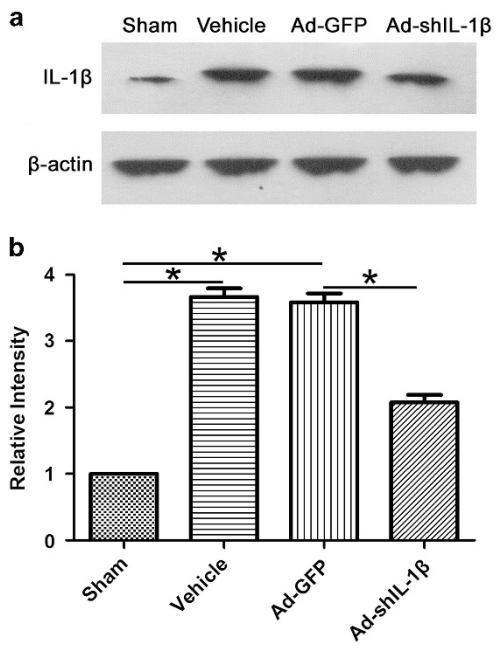

Figure 1 Detection of IL-1β protein in the spinal cords. (**a**) IL-1β and β-actin detected by western blot in the spinal cord lysates obtained from four groups 24 h after SCI. The blots are representative examples from six experiments each group. (**b**) The quantitative mean ± s.d. data show the level of IL-1β protein expression. The band density is normalized as the ratio of IL-1β:β-actin ($n = 6$). *$P < 0.05$.

the Ad-shIL-1β group were significantly lower compared with the levels in the Vehicle and the Ad-GFP groups ($P<0.05$).

Neurological outcomes

The evaluations of the hindlimb locomotor functions at pre-injury and 1, 3, 7, 14 and 21 days after SCI were shown in Figure 2. All the rats initially showed a slight decrease in the BBB score, and there was no significant difference among the four groups before SCI. The Sham group showed full recovery 3 days after SCI. The Vehicle, the Ad-GFP and the Ad-shIL-1β groups showed a sharp decrease in the BBB score 1 day after SCI, and there was no significant difference among the three groups. Partial improvements were observed, and there was no significant difference among the Vehicle, the Ad-GFP and the Ad-shIL-1β groups 3 days after SCI. However, the neurological improvements were significantly greater in the Ad-shIL-1β group compared with the improvements in the Vehicle and the Ad-GFP groups at 7, 14 and 21 days after SCI ($P<0.05$).

Ad-shIL-1β increases neuronal survival after SCI

Neuron survival was examined by an immunofluorescence technique using a specific antibody for neuron (neuN) (Figure 3). Qualitatively, neuron density was decreased in the lesion sections of the Vehicle group, the Ad-GFP group and the Ad-shIL-1β group when compared with the corresponding sections of the Sham group. However, Ad-shIL-1β significantly attenuated the decrease in neuron survival compared with vehicle animals.

TNF-α expression after SCI

The TNF-α levels in the spinal cord 24 h after SCI were shown in Figure 4. The levels of TNF-α in the Sham group were significantly lower compared with the levels in the Vehicle, the Ad-GFP and the Ad-shIL-1β groups ($P<0.05$). However, the levels of the TNF-α in the Ad-shIL-1β group were significantly lower compared with the levels in the Vehicle and the Ad-GFP groups ($P<0.05$).

Bax, Bcl-2 and active caspase-3 protein detection

Figures 5–7 show the western blot analysis of Bax, Bcl-2 and active caspase-3 in all groups of animals 24 h after SCI. The expressions of Bax and active caspase-3 were low in the spinal cord of the Sham group. In contrast, the expression of Bcl-2 was high. SCI induced increased expressions of Bax and active caspase-3 and decreased expression of Bcl-2. Ad-shIL-1β treatment greatly reduced the expressions of Bax and active caspase-3 and increased the expression of Bcl-2.

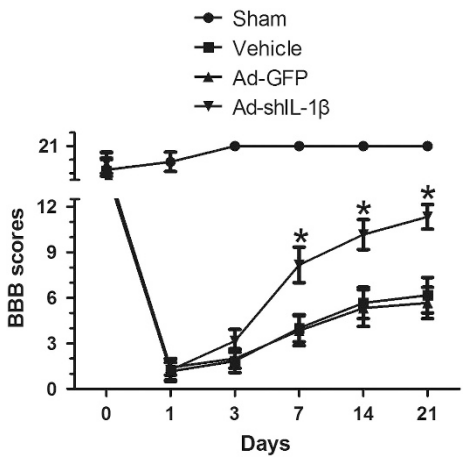

Figure 2 The hindlimb functional assessment with the BBB rating scale at pre-injury and 1, 3, 7, 14 and 21 days after SCI in each group. The data were plotted as the mean ± s.d. $^*P<0.05$.

Ad-shIL-1β decreases leukocyte infiltration in the spinal cord

To further evaluate the post-SCI inflammation in each group, we isolated infiltrating leukocytes from spinal cord segments with lesions and analyzed distinct leukocyte populations using flow cytometry. Here, we did not analyze the vehicle group because the data-mentioned above indicated that there was no remarkable difference between the vehicle and the Ad-GFP groups. As shown in Figure 8, the number of Gr1+ granulocytes at day 1 after SCI in Ad-GFP group was significantly higher in comparison with the sham group, and, Ad-shIL-1β profoundly reduced the granulocyte number, suggesting inhibition of granulocyte infiltration. At day 7 after SCI, the number of CD45loCD11b+ microglia was not significantly changed among each group. The numbers of CD45hiCD11b+ macrophages and CD3+ T cells were remarkably increased in the Ad-GFP group, compared with the sham group. However, their numbers were significantly decreased in the Ad-shIL-1β group in comparison with the Ad-GFP group, suggesting inhibition of infiltration of

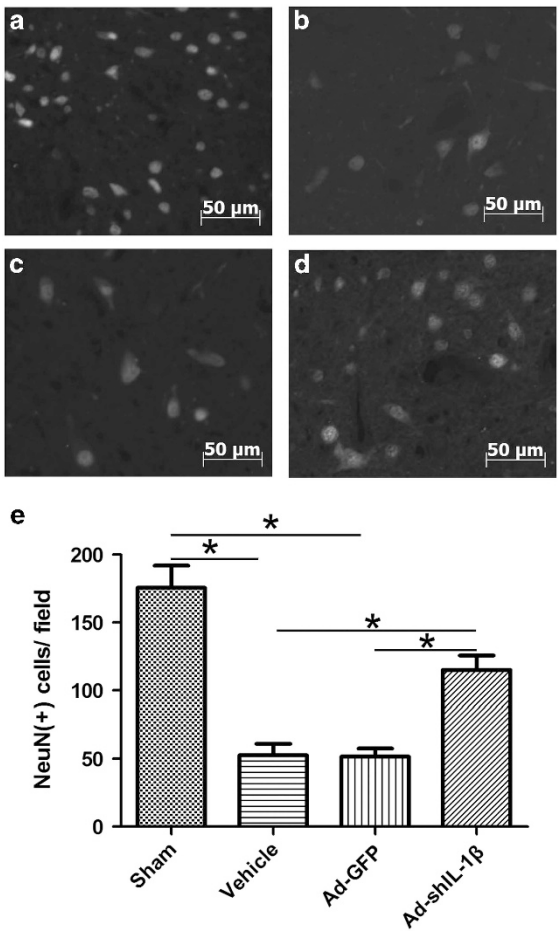

Figure 3 After NeuN staining, six sections were randomly selected from each animal, and six microscopy fields of each section were randomly selected for quantification of NeuN+ cells. A total of six rats were analyzed in each group. The density of neurons at day 21 after SCI in the Sham (**a**), Vehicle (**b**), Ad-GFP (**c**) and Ad-shIL-1β (**d**) groups was determined by immunofluorescent labeling with anti-neuN antibody. (**e**) The data were plotted as the mean ± s.d. $^*P<0.05$. A full color version of this figure is available at the *Spinal Cord* journal online.

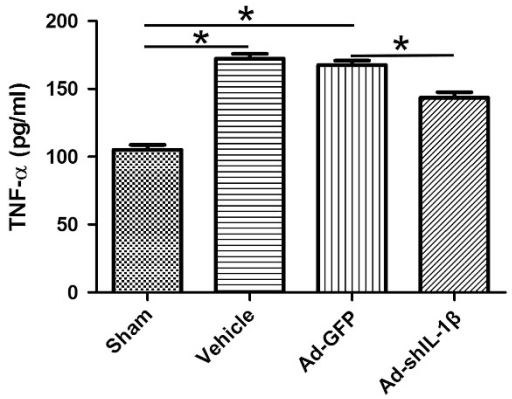

Figure 4 The TNF-α levels in the spinal cord tissues 24 h after SCI. Spinal cord homogenates from six rats of each group were used. Each sample was triplicated in the 96-well enzyme-linked immunosorbent assay (ELISA) plate. A standard curve was generated by applying the standard TNF-α protein provided in the kit. The data were plotted as the mean ± s.d. $*P<0.05$.

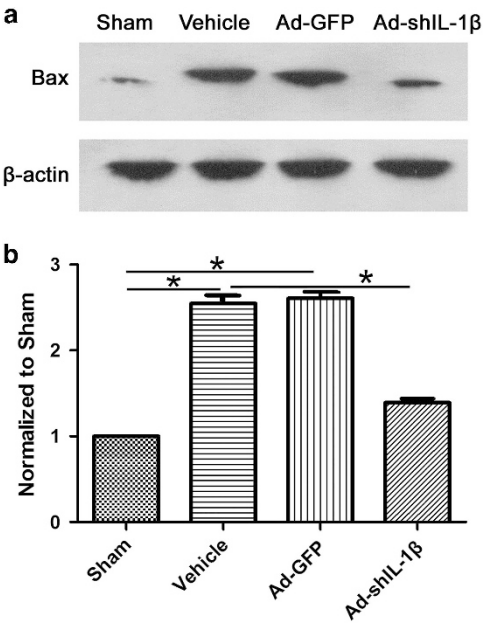

Figure 5 Detection of Bax protein in the spinal cords. (**a**) Bax and β-actin detected by western blot in the spinal cord lysates obtained from four groups 24 h after SCI. The blots are representative examples from six experiments each group. (**b**) The quantitative mean ± s.d. data show the level of Bax protein expression. The band density is normalized as the ratio of Bax:β-actin ($n=6$). $*P<0.05$.

macrophages and T cells. Taken together, our data indicated that Ad-shIL-1β restrained infiltration of inflammatory leukocytes into post-SCI spinal cords.

GFAP detection

To evaluate the astrocyte reaction after SCI, we detected GFAP expression in the injured spinal cord sections using western blot. We found that GFAP expression was increased by about two-folds after SCI, and there was no significant difference of GFAP expression between the Ad-GFP and the Ad-shIL-1β groups (Figure 9), suggesting that Ad-shIL-1β might not influence reactive astrocytes after SCI.

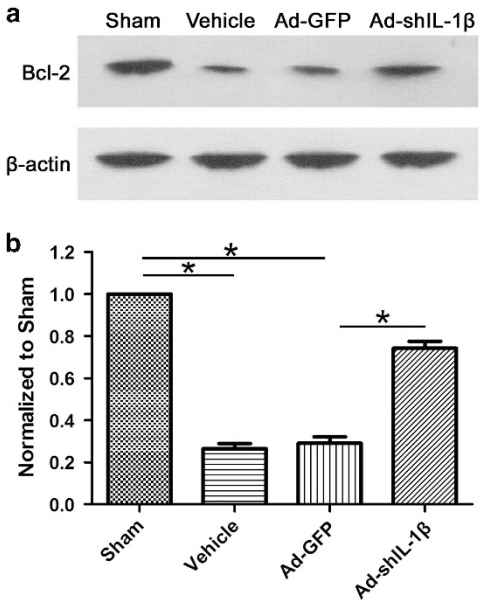

Figure 6 Detection of Bcl-2 protein in the spinal cords. (**a**) Bcl-2 and β-actin detected by western blot in the spinal cord lysates obtained from four groups 24 h after SCI. The blots are representative examples from six experiments each group. (**b**) The quantitative mean ± s.d. data show the level of Bcl-2 protein expression. The band density is normalized as the ratio of Bcl-2:β-actin ($n=6$). $*P<0.05$.

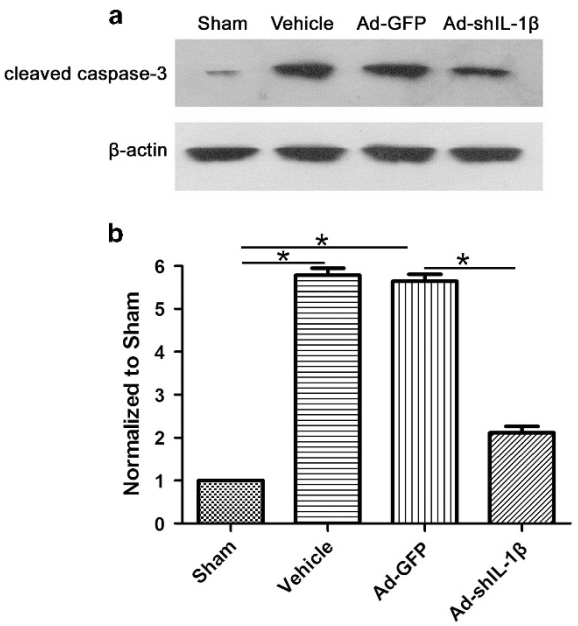

Figure 7 Detection of caspase-3 protein in the spinal cords. (**a**) Caspase-3 and β-actin detected by western blot in the spinal cord lysates obtained from four groups 24 h after SCI. The blots are representative examples from six experiments each group. (**b**) The quantitative mean ± s.d. data show the level of caspase-3 protein expression. The band density is normalized as the ratio of caspase-3:β-actin ($n=6$). $*P<0.05$.

DISCUSSION

It is important to develop a therapy that can reduce the evolution of the secondary damages in injured spinal cords. A number of recent studies have focused on the effects of gene therapies on secondary injury.[22] The data from the present study demonstrated a protective

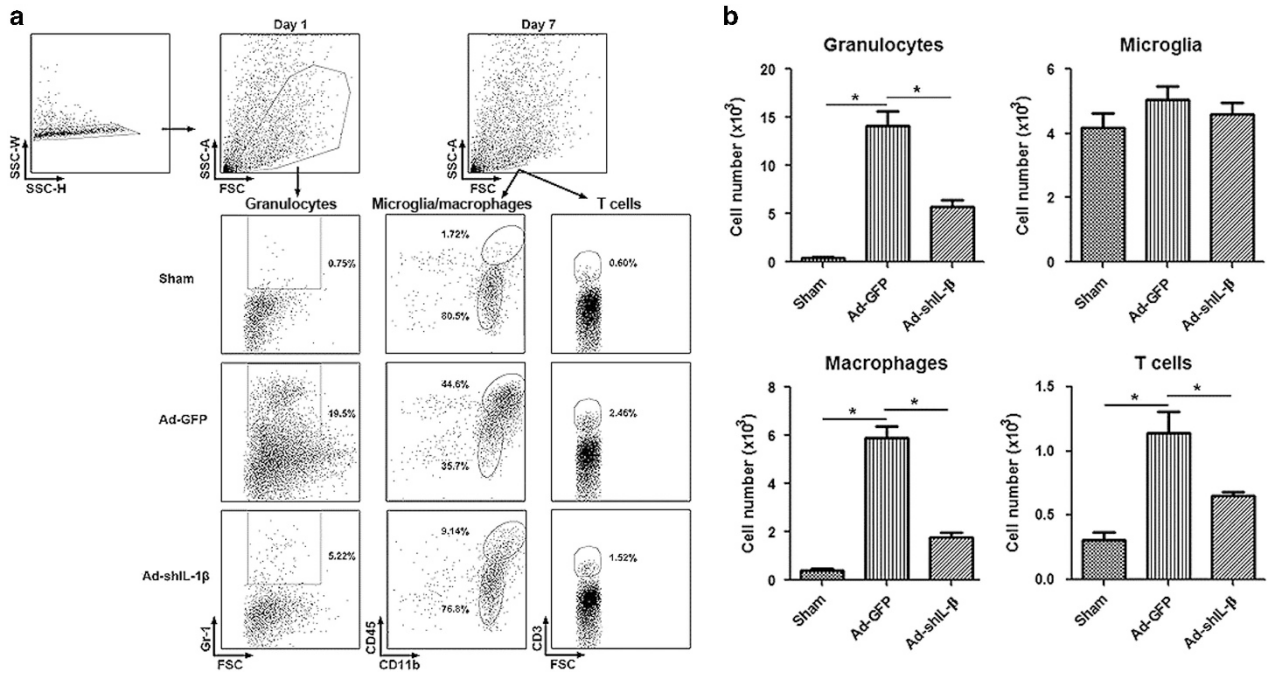

Figure 8 Ad-shIL-1β decreases leukocyte infiltration in the spinal cord. (**a**) Flow-cytometric gating strategy for detection of leukocyte populations in isolated spinal cord cells. Single cells were selected using side scatter-height (SSC-H) and scatter-width (SSC-W). Then, Forward scatter (FSC) and side scatter-area (SSC-A) were used to gate total leukocytes. Gr1+ granulocytes were gated in the total leukocytes isolated from spinal cords at 24 h after SCI. For detection of microglia/macrophages and T cells, total leukocytes isolated from spinal cords at 7 days after SCI. CD45hiCD11b+ macrophages, CD45loCD11b+ microglia and CD3+ T cells were gated, respectively. Numbers in the dot plots are the percentages of corresponding cell populations. (**b**) Cell number of each leukocyte population in the spinal cord after SCI. $*P<0.05$.

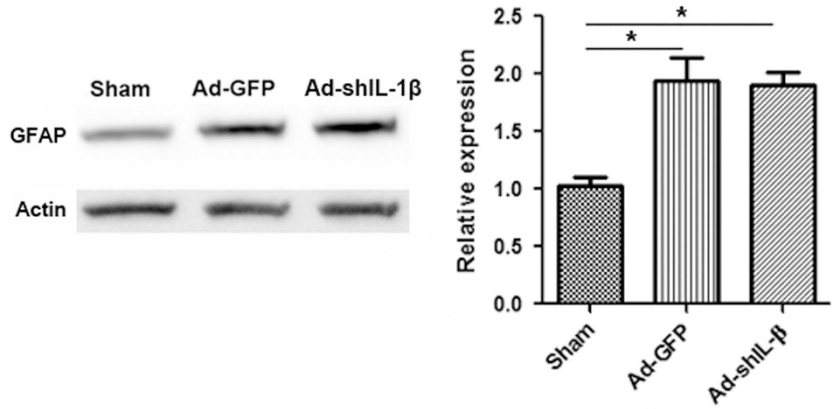

Figure 9 Ad-shIL-1β does not influence astrocyte reaction to SCI. Expression of GFAP in the spinal cord segments with lesions that were harvested at 24 h after SCI. Left panel: representative western blot image. Right panel: Statistics for relative expression of GFAP. $*P<0.05$.

effect of the adenovirus-mediated shRNA against IL-1β gene on traumatic SCI.

The gene transfer of specific genes for therapeutic purposes offers a valuable approach to the treatment of SCI.[22] It has been proven that adenoviruses efficiently transfer exogenous gene expression in the central nervous system including the spinal cord.[23] Therefore, in the present study, we used an adenoviral vector expressing shRNA to IL-1β, followed by transfection into the spinal cords of the rats *in vivo*. As a result, the downregulation of IL-1β in the spinal cords promotes function recovery and increases neuronal survival after SCI.

Secondary injury in traumatic SCI is believed to be a result of a several destructive process such as inflammation, apoptosis, ischemia, hemorrhage and edema, all of them can cause dysfunction and death in neuronal cells.[6] Previous reports stated that one of the most important factors precipitating post-traumatic degeneration in the spinal cord is inflammation.[24] Furthermore, apoptosis is believed to have an important role in the pathogenesis of secondary injury.[25] Consequently, reducing inflammation or apoptosis may improve neurological recovery or facilitate nerve regeneration.

TNF-α is a major initiator of inflammation and is released early after SCI.[26] There is evidence that TNF-α also has an important role in the recruitment of inflammatory and immune cells to the injured site.[27] Moreover, the infiltration of inflammatory cell to SCI sites is a major contributor to secondary degeneration.[28] The inhibition of

TNF-α promotes functional recovery after a traumatic SCI.[29] The results presented in this study indicated that the TNF-α levels increased significantly in the spinal cord tissues 24 h after SCI. However, the IL-1β downregulation could significantly attenuate the increase in the TNF-α level, due to its ability to reduce inflammation. Consistent with the decreased pro-inflammatory cytokine levels in the spinal cord, our data indicated that Ad-shIL-1β significantly reduced leukocyte infiltration into the spinal cord after SCI. As an index of inflammation, inflammatory leukocyte infiltration has a critical role in the secondary damage of central nervous system tissues. Therefore, Ad-shIL-1β might alleviate the detrimental effect of inflammatory leukocytes through inhibition of their recruitment into the spinal cord. In addition, we did not observe significant changes in reactive astrocytes, suggesting that astrocyte reaction to SCI might not be tightly regulated by IL-1β. However, we do not know whether astrocyte-derived cytokines were altered by application of Ad-shIL-1β. Our ongoing study is checking the expression of both pro- and anti-inflammatory cytokines, as well as neurotrophic factors, in astrocytes after SCI.

Bcl-2 family proteins are the key step on regulation of apoptosis in the nervous system,[30] which are composed of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins that regulate the mitochondrial pathway of programmed cell death.[31] Bcl-2 is the best-understood protein in a cell death pathway, inhibiting apoptosis and extending cell survival.[32] Bax is a protein that promotes cell death by forming a heterodimer with Bcl-2 and blocking its anti-apoptotic actions.[33] Caspases, especially caspase-3, are known to act downstream of Bax/Bcl-2 control and have a key role in the execution of apoptosis.[34] In the present study, we detected the expressions of Bcl-2, Bax and cleaved-caspase-3 at 24 h after SCI and found that SCI upregulated the expressions of Bax and active caspase-3, and downregulated the expression of Bcl-2, which were consistent with the findings from previous studies.[18,35] However, adenovirus-mediated shRNA against IL-1β gene on traumatic SCI suppressed apoptosis by decreasing the expressions of Bax and active caspase-3, and increasing the expression of Bcl-2, thereby reducing cell death, and protected the injured spinal cord neurons.

CONCLUSION

In summary, the present study shows that IL-1β downregulation clearly had the ability to ameliorate inflammation and prevent apoptosis after the traumatic SCI. Our results indicated that the IL-1β downregulation may have potential therapeutic benefits not only for reducing the secondary damages but also for improving the outcomes after traumatic SCI.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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