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JNK activation contributes to DP5 induction and apoptosis following traumatic spinal cord injury

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Growing evidence suggests that cells undergo apoptosis after spinal cord injury (SCI). However, little is known about the early events that trigger apoptosis in the contused cord. The BH3-only subfamily of pro-apoptotic regulators (e.g., *bim*, *bad*, and *dp5*) is recognized as initiators of the apoptotic cascade, and is subject to stringent control, both at the transcriptional and post-translational level. In the current study, we studied upstream events regulating trauma-induced apoptosis in the spinal cord. Within 1 h after SCI in rats, DP5 was induced, while Bim and Bad levels remained unchanged. In parallel, SCI also activated the stress-induced c-Jun N-terminal kinase (JNK), leading to the phosphorylation of c-Jun, with a similar temporal profile. Immunohistochemical analysis revealed that p-JNK and DP5 colocalized to neurons and oligodendrocytes undergoing apoptosis in the injured cord, but were absent in uninjured spinal cord. Furthermore, inhibition of JNK activity with in vivo delivery of SP600125 or a *jnk1* antisense oligodeoxynucleotide (ODN) attenuated DP5 induction and caspase-3 activation. These results suggest that JNK activation contributes to trauma-induced DP5 expression and subsequent apoptosis in SCI.

Keywords: Apoptosis; BH3-only family; Caspase 3; DP5; JNK; Spinal cord injury

Introduction

Trauma to the spinal cord results in the loss of motor and sensory function distal to the point of injury and devastating disability predominantly affecting young adults (Bracken et al., 1981). Accumulating evidence suggests that neural cells die with features of apoptosis following SCI. These apoptotic characteristics have included ultrastructural features (shrunken, fragmented nuclei, apoptotic bodies) and biochemical markers such as DNA laddering (Hamada et al., 1996; Crowe et al., 1997; Liu et al., 1997; Emery et al., 1998). In addition, the specific apoptotic enzymes or their end-products were detected in the contused cord. These include the activated form of caspase 3, and cleaved products of several members of the inhibitors of apoptosis (IAP) family (Springer et al., 1999; Li et al., 2000; Keane et al., 2001; Kim et al., 2001). Evidence that apoptosis following SCI is of potential clinical significance comes from studies that inhibition of apoptosis by cyclohexamide (Liu et al., 1997) or a broad-spectrum caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Li et al., 2000), reduced the lesion size and improved motor function in mice after SCI. Apoptosis in cells derived from the nervous system appears to be dependent on new RNA and protein synthesis, as inhibitors of these processes block apoptosis (Martin et al., 1988). Subsequent studies suggest that the selective expression of genes in the *bcl-2* family of apoptotic regulators were responsible for the transcription-dependent initiation of apoptosis. In particular, a subgroup of the Bcl-2 family proteins, which share the BH3 homology domain, were implicated (Putcha et al., 2001; Whitfield et al., 2001). These "BH3-only" proteins, including Bad, Bak, Bid, Bim, and DP5, translocate to mitochondria from other cellular compartments and interact with other Bcl-2 members to regulate mitochondrial release of pro-apoptotic proteins (Kelekar and Thompson, 1998; Huang and Strasser, 2000). BH-3-only proteins are stringently regulated at the transcriptional and post-translational levels during apoptosis, depending on the cell type and apoptotic stimulus (Bouillet et al., 1999; Dijkers et al., 2000; Huang and Strasser, 2000; Whitfield et al., 2001). Several of the BH3-only genes including *dp5* (Imaizumi et al., 1997; Inohara et al., 1997) and *bim* (Putcha et al., 2001; Yin et al., 2002) are subject to transcriptional control.

The c-Jun N-terminal kinase (JNK) represents a group of mitogen-activated protein kinases (MAPKs) that mediate stress-induced cellular events including apoptosis (Davis, 2000). The JNK signaling pathway plays a critical role in mediating apoptosis in the developing and mature organisms via transcription-dependent and transcription-independent mechanisms that remain incompletely understood. JNK promotes cell death by directly regulating the cell death machinery (Ito et al., 1997; Maundrell et al., 1997; Srivastava et al., 1999; Yamamoto et al., 1999; Fan et al., 2000;

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Tournier et al., 2000). For example, JNK has been reported to catalyze the phosphorylation of Bcl-2 to suppress the anti-apoptotic function of Bcl-2 (Srivastava et al., 1999; Yamamoto et al., 1999). In addition, JNK has been found to catalyze the phosphorylation of Bad, a BH3-only protein, to promote its pro-apoptotic effect in primary cultures of neurons (Bhakar et al., 2003). More recent reports indicate that JNK may also mediate transcription-dependent apoptosis by inducing the BH3-only genes, including *dp5* and *bim* (Yang et al., 1997a; Davis, 2000; Harris and Johnson, 2001; Putcha et al., 2001, 2003; Whitfield et al., 2001; Yin et al., 2002).

Since apoptosis of neural cells after SCI has been characterized, we explored the early events leading to apoptosis after SCI. In particular, we examined the transcriptional regulation of the BH3-only family members by upstream signaling kinases after SCI to explore potential therapeutic targets.

Materials and methods

Spinal cord injury model in rats

Female Long–Evans rats (body weight 300 ± 25 g; Simonsen Laboratories, Gilroy, CA) were used in this study. All surgical procedures and animal experimentation protocols followed the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the Guidelines and Policies for Rodent Survival Surgery approved by the Animal Studies Committee of Washington University School of Medicine. The method for inducing SCI followed the Multicenter Animal Spinal Cord Injury Study (MASCIS) protocol as reported previously (Basso et al., 1996) with modifications. Briefly, rats were anesthetized with intraperitoneal injection of chloral hydrate (300–400 mg/kg) and a laminectomy was made at the T9–10 segment. During surgery, rectal temperature was monitored and maintained at 37.0 ± 0.5°C by a heating pad. Using a New York University (NYU) Impactor, SCI was induced by dropping a 10-gm weight at a height of 12.5 mm (Gruner, 1992). After injury, the muscles and incision were closed in layers, and the animals were placed in a temperature- and humidity-controlled chamber (Thermocare, Incline Village, NV) for 3 h. Manual bladder expression was performed three times per day until reflex bladder emptying was established. Animals subjected to identical surgical procedures without injury served as sham-operated controls. In addition, animals that received no surgery were also included as normal controls.

Treatment protocols

Rats (n = 6–7 per group) were treated with an antisense or mismatch phosphorothioated oligodeoxynucleotide (ODN) to jnk1. The ODNs were custom-made by Molecular Research Laboratories (Herndon, VA) using the following sequences: 5′-CTCATGATGG-CAAGCAATTA-3′ (antisense) and 5′-GCTCGGTGGAAATG-GATCAG-3′ (mismatch). The antisense or mismatch ODN in a volume of 2.5 μl (2 μl of 1 μM ODN in PBS, pH 7.4, mixed with 0.5 μl of 1 mg/ml lipofectin) was infused into the rat spinal cord directly with the needle tip at the depth of 1.5 mm from the dorsal midline surface under the guidance of a stereotaxic device 16 h before injury. The infusion rate was 1 μl/min, and the needle was withdrawn 5 min after completion of ODN delivery. An identical volume of the vehicle (2 μl PBS mixed with 0.5 μl of 1 mg/ml lipofectin) was infused in another set of control animals. For treatment with a JNK inhibitor, SP600125 was administrated 12 h prior to injury at a dose of 15 mg/kg s.c. (n = 6–7 per group) in PPCES vehicle (30% PEG-400/20% polypropylene glycol/15% Cremophor EL/5% ethanol/30% saline) (Bennett et al., 2001).

Western blotting

This method has been reported previously (Kim et al., 2001; Yin et al., 2002). Briefly, at various intervals post-injury (n = 3–4 per time point), a 10-mm spinal cord segment (5 mm rostral and 5 mm caudal from the epicenter) was dissected following intracardiac perfusion with 200 ml saline following pentobarbital anesthesia (100 mg/kg, i.p.) and frozen immediately in liquid nitrogen. The cord segment was homogenized in buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin; pH 7.9) and centrifuged at 14,000 × g. An equal amount of proteins (40–60 μg) from the supernatant of each sample was loaded onto a 10–15% polyacrylamide gel depending on target protein molecular weight, separated by SDS/PAGE, and transferred to PVDF membranes by electrophoresis. The membranes were blocked in TBST buffer containing 20 mM Tris–HCl, 5% nonfat milk, 150 mM NaCl, and 0.05% Tween-20 (pH 7.5) for 1 h at room temperature. Primary rabbit anti-JNK antibody, rabbit anti-p-JNK antibody, rabbit anti-c-Jun antibody, rabbit anti-p-c-Jun (Ser73) antibody, rabbit anti-p-c-Jun (Ser63) antibody, rabbit anti-Bad antibody (1:1000; Cell Signaling, MA), rabbit anti-DP5 antibody (1:200; custom-made by Bethyl Laboratories INC, Montgomery, Texas), rabbit anti-Bim antibody (1:1000, Stressgen, Victoria, BC, Canada), rabbit anti-active caspase 3 antibody (1:2000, BD Biosciences, San Diego, CA), or mouse anti-actin antibody (1:400; Santa Cruz, CA) was added to the membrane and incubated for 2 h at room temperature. The membrane was washed with TBST three times at 10-min intervals, incubated with the second antibodies (1:3000–5000; rabbit, mouse, or rat IgG conjugated with alkaline phosphatase) for 1 h, then washed 3 times each at 10-min interval with TBST and 2 times each for 2 min with TBS. The color reaction was developed using the Blot AP System as described in the technical manual provided by Promega (Madison, WI). Quantitative analysis of selected bands in Western blots was performed by using the NIH Image Analysis System.

Immunohistochemical staining

The rats (n = 3 per group) were overdosed by intraperitoneal injection of pentobarbital sodium (100 mg/kg). Intracardial perfusion fixation was carried out first with saline followed by 300 ml of 4% paraformaldehyde in 0.1 M PBS. The spinal cord was dissected out and a 10-mm segment containing the injured epicenter was blocked, post-fixed for 2 h in the same fixative solution, and transferred to a solution containing 30% sucrose in 0.1 M PBS (pH 7.4) sequentially for diaminobenzidine (DAB) or immunofluorescent staining. The 10-mm cord segments including the lesion epicenter was then embedded in tissue freezing medium (Sakura Finetek U.S.A., Torrance, CA), cut horizontally or longitudinally at a thickness of 20–30 μm on a cryostat, and mounted on Superfrost/plus slides (Fisher Scientific, Pittsburgh, PA). Before incubation with primary antibodies, the sections were permeabilized with 0.3% Triton X-100/10% normal goat serum in 0.01 M PBS for 60 min. Mouse anti-p-JNK antibody (1:100; Cell

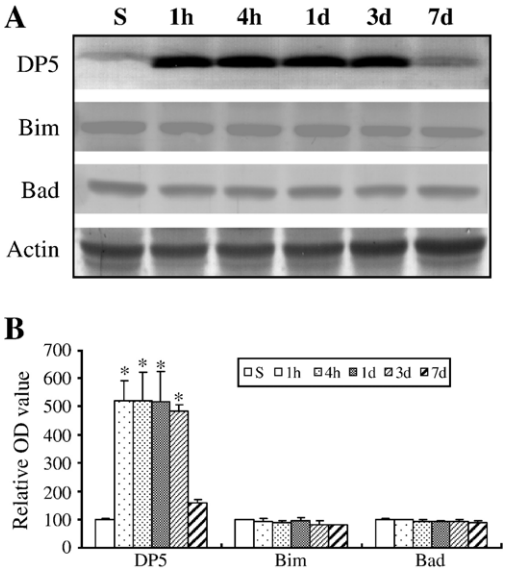

Fig. 1. Expression of the BH3-only proteins after SCI: (A) 10 mm spinal cord segments were extracted and prepared for Western blotting at various times after SCI using anti-DP5, anti-Bim, anti-Bad, and anti-actin antibodies. DP5, which was absent in the uninjured cord, increased dramatically, starting 1 h after injury and persisting for 3 days before declining to basal levels after 7 days. No changes in Bim or Bad expression were observed; note that both Bim and Bad were present in the uninjured cord. Depicted is a representative blot from 3 rats with similar results. (B) Quantitative analysis of three Western blots (normalized to actin) using the NIH Image Analysis System graphically illustrated an increase in DP5 level after SCI in a time dependent manner. Data in relative density are expressed as mean ± SD. *$P < 0.05$ versus sham group.

Signaling, MA) or rabbit anti-DP5 antibody (1:40; Custom-made by Bethyl Laboratories INC, Montgomery, Texas) was then applied to the sections overnight at 4°C. The following day, sections were incubated with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA), or horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 h. After washing, sections were incubated with ABC Elite complex (1:100; Vector Laboratories, Burlingame, CA) for 1 h. The staining was visualized with DAB for 2–5 min (Sigma, St. Louis, MO). Slides were washed, dehydrated, cleared in xylene, and coverslipped. Slides were examined with Olympus BX60 light microscope (Olympus Optical, Tokyo, Japan). In negative control sections, the primary antibodies were substituted by normal goat serum in 0.01 M PBS.

Immunofluorescence double labeling

Before primary antibody incubation, the spinal cord sections from rats ($n = 3–4$ per group) were permeabilized with 0.3% Triton X-100/10% normal goat serum in 0.01 M PBS for 30 min. Polyclonal or monoclonal anti-p-JNK antibody (1:100; Cell Signaling, MA), mouse anti-NSE antibody (1:250; Chemicon, Temecula, CA), mouse anti-PLP antibody (1:100; Chemicon, Temecula, CA), mouse anti-GFAP antibody (1:200; Chemicon), rabbit anti-DP5 antibody (1:40; Custom-made by Bethyl Laboratories INC, Montgomery, Texas), and/or rabbit anti-active caspase 3 antibody (1:100; Pharmingen, San Jose, CA) were applied to the sections overnight at 4°C. On the following day, the sections were incubated with fluorescein-conjugated goat anti-rabbit antibody (1:100; Vector Laboratories, Burlingame, CA) and Texas-Red-conjugated goat anti-mouse antibody (1:100; Vector Laboratories, Burlingame, CA) for 1 h. Slides were washed, wet mounted and examined under a Zeiss fluorescence microscope.

Statistical analysis

Quantitative data were expressed as Mean ± SD values. Difference among groups was statistically analyzed by one-way analysis of variance followed by Bonferroni's post hoc test. Comparison between two experimental groups was based on a two-tailed *t* test. A *P* value less than 0.05 was considered significant.

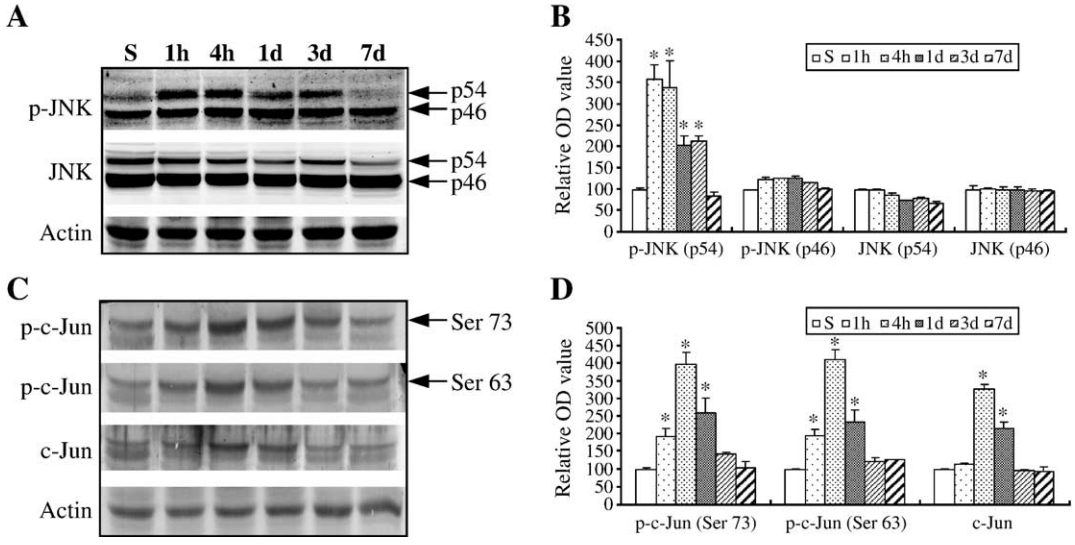

Fig. 2. Time-dependent activation of JNK and c-Jun phosphorylation after SCI: Spinal cord segments from contused cords were prepared for Western blotting at indicted times after SCI, using anti-JNK, anti-p-JNK, anti-c-Jun, and anti-p-c-Jun antibodies. P-JNK at p54 increased rapidly after SCI, persisting for 3 days before returning to basal levels. SCI had no effect on total JNK levels (A, B). SCI increased the expression of c-Jun and p-c-Jun with a similar time course (C, D). As internal controls, blots were also analyzed by immunoblotting with anti-actin antibody. Data shown in panels A, C are representative blots from rats with similar results. Data in relative density in panels B, D are expressed as mean ± SD. *$P < 0.05$ versus sham group.

Results

Expression of BH3-only family members after SCI

In order to identify BH3-only family members that are induced after SCI, we examined the expression of several candidate proteins, including DP5, Bim, and Bad at various times after SCI. Of the proteins examined, SCI induced the expression of DP5 only as early as 1 h post-injury, persisting for 3 days before declining to basal levels at 7 days post-injury. No significant changes in Bim or Bad expression were noted during this time period (Figs. 1A, B).

JNK and c-Jun phosphorylation after SCI

The JNK signaling pathway appears to play a critical role in mediating apoptosis via transcription-dependent mechanisms, involving the phosphorylation of c-Jun and the consequent expression of target apoptotic genes (Estus et al., 1994; Xia et al., 1995; Yang et al., 1997a; Watson et al., 1998; Behrens et al., 1999; Le-Niculescu et al., 1999). We sought to determine if the JNK signaling pathway was activated early after SCI. Western blotting revealed low basal levels of p-JNK protein expression in sham-operated controls. SCI caused a substantial increase in p-JNK expression in a time-dependent manner, starting at 1 h, and returning to basal levels 7 days after injury (Figs. 2A, B). Interestingly, JNK phosphorylation occurred primarily at p54, while p46 remained unchanged. There was no significant change in total JNK protein during this time period. The transcription factor, c-Jun (which is an exclusive substrate of JNK), was also phosphorylated shortly after traumatic injury, showing a similar time-course as p-JNK (p54) following SCI (Figs. 2C, D). These data suggest that SCI induces activation of JNK and phosphorylation of its substrate, c-Jun, shortly after impact, and raise the possibility that this pathway may contribute to SCI-induced apoptosis.

Co-localization of p-JNK and DP5 in apoptotic cells following SCI

JNK may also mediate transcription-dependent apoptosis by inducing the BH3-only genes, *dp5* and *bim* (Yang et al., 1997a; Davis, 2000; Harris and Johnson, 2001; Putcha et al., 2001, 2003;

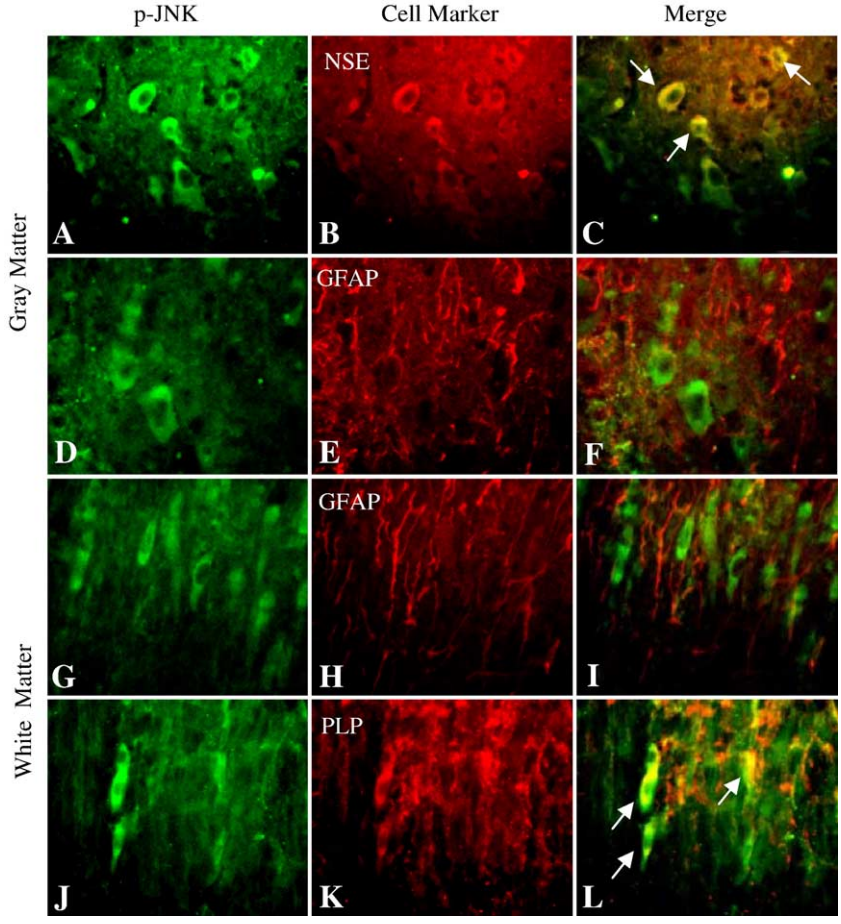

Fig. 3. Increased p-JNK immunoreactivity in neurons and oligodendrocytes after SCI: Longitudinal sections of contused spinal cord 1 mm rostral to the lesion epicenter (24 h after SCI) were prepared for immunohistochemistry using anti-p-JNK (FITC, green, A, D, G, J), anti-NSE (Texas red, B), anti-GFAP (Texas red, E, H), or anti-PLP antibody (Texas red, K), as indicated. p-JNK immunoreactivity (A) colocalized with NSE immunoreactivity (B), suggesting neuronal expression of p-JNK (C, merged) in the gray matter. Likewise, p-JNK immunoreactivity (J) colocalized with the oligodendrocyte marker, PLP (K, L, merged). No colocalization of p-JNK (D, G) and the astrocytes-specific antigen, GFAP, was detected in both white matter (D–F) and gray matter (G–I). Arrows indicate cells demonstrating colocalization of p-JNK and respective cell-specific markers. p-JNK immunostaining was absent in uninjured cord (data not shown). Magnification: A–C, 200×; D–L, 400×.

Whitfield et al., 2001; Yin et al., 2002). The expression of DP5, but not Bim, was upregulated following SCI. If JNK activation results in DP5 induction and apoptosis after SCI, then p-JNK should colocalize with DP5 in apoptotic cells in the injured cord. Using immunohistochemistry, we examined the expression of p-JNK and DP5 in injured and uninjured spinal cords. Both p-JNK and DP5 immunoreactivity was absent in sham-operated controls (data not shown). One day after injury, p-JNK immunofluorescent staining was observed in cells in the gray matter and in the lateral funiculus adjacent to the injury epicenter (Fig. 3). p-JNK expression appeared to extend from the injury epicenter to rostral and caudal cord segments (data not shown). In the gray matter, many cells with the morphological features characteristic of neurons in the ventral horn and intermediate zone showed strong p-JNK immunoreactivity (Figs. 3A, D). Indeed, double-labeling with neuron-specific enolase (NSE) demonstrated that virtually all p-JNK-positive cells exhibited NSE immunoreactivity in the gray matter (Fig. 3C). In the adjacent white matter, many oligodendrocyte-like cells stained p-JNK-positive as well, which formed longitudinal rows in the white matter in the long axis of the spinal cord (Figs. 3G, J). This result was confirmed by double-labeling with an antibody against PLP, an oligodendrocyte-specific marker (Fig. 3L). There was no double-labeling of p-JNK-positive cells with an antibody against glial fibrillary acidic protein (GFAP) in either white or gray matter of the contused cord (Figs. 3F, L).

To determine if p-JNK and DP5 co-localized to the same neural cells after SCI, we double-labeled injured cords with respective antibodies. p-JNK and DP5 immunoreactivity colocalized to the same cells in both white matter (Figs. 4A–C) and gray matter (Figs. 4D–F). Furthermore, p-JNK also colocalized with active caspase 3 immunoreactivity in neural cells of the white and gray matter (Figs. 4G–L). In control sections incubated with normal mouse IgG in the absence of the primary antibody for p-JNK, there was no labeling (data not shown). These data suggest that after SCI, activated JNK is present in cells undergoing apoptosis.

Attenuation of DP5 upregulation, and caspase 3 activation following SCI by JNK inhibition

To determine if JNK activity mediates SCI-induced DP5 upregulation, we used two strategies to inhibit JNK activity after SCI. The first pharmacological approach involved the subcutaneous administration of a specific JNK inhibitor, SP600125, 12 h prior to SCI. Four hours after SCI, p-JNK levels were increased in

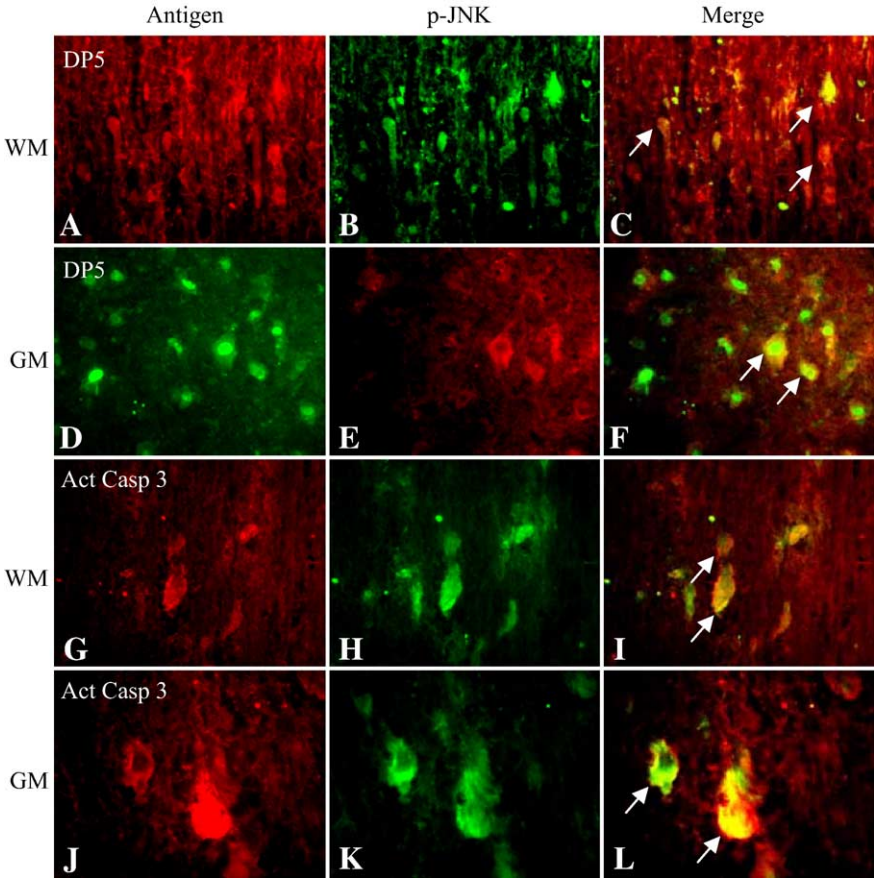

Fig. 4. Co-localization of p-JNK with DP5 and activated form of caspase 3 after SCI: Longitudinal sections of contused spinal cord 1 mm rostral to the lesion epicenter (24 h after SCI) were prepared for immunohistochemistry using anti-p-JNK (B, E, H, K), anti-DP5 (A, D), or anti-active caspase 3 (Act Casp 3) antibodies (G, J), as indicated. p-JNK immunoreactivity (B, E) colocalized in cells immunoreactive to anti-DP5 antibodies (A, D) in the gray and white matter (C, F, merged). p-JNK immunoreactivity (H, K) also colocalized with that of the activated form of caspase-3 (G, J) in the gray and white matter (I, L, merged). No immunoreactivity of DP5 or the activated form of caspase 3 was detected in uninjured cords (data not shown). Arrows indicate cells exhibiting immunoreactivity of double antigens. Magnification: A–C, 200×; D–L, 400×.

vehicle treated animals, but this increase was suppressed in animals pretreated with SP600125 (Figs. 5A, B). Furthermore, SCI-induced DP5 expression was suppressed by SP600125, as was caspase 3 activation, suggesting that JNK activity mediated DP5 expression and subsequent caspase 3 activation and apoptosis (Figs. 5A, B).

To confirm the results obtained with SP600125, a previously established antisense strategy was used (Liu et al., 1994; Cui et al., 1999; Xu et al., 2001). An antisense ODN specific for *jnk1* (or a mismatched ODN serving as a control) was infused into the spinal cord 16 h prior to SCI. As illustrated in Figs. 5C, D, in vivo transfection of this *jnk1* antisense ODN effectively reduced post-traumatic JNK phosphorylation, resulting in decreased induction of DP5 and attenuation of caspase 3 activation after SCI. The infusion of a mismatch ODN had no effect on JNK activation, DP5 expression or caspase 3 activation, illustrating the specificity of this *jnk1* antisense approach (Figs. 5C, D).

Discussion

SCI produces a predictable pattern of progressive injury resulting in neuronal and glial cell death, vascular injury, axonal destruction, and demyelination (Bresnahan, 1978; Blight, 1985; Dusart and Schwab, 1994). Cell death after SCI appears to be mediated by several mechanisms, including energy loss, electrogenic pump failure, and membrane depolarization, glutamate excitotoxicity, intracellular calcium overload, excessive generation of free radicals and inflammatory reaction (Jacobs et al., 1987; Faden et al., 1989; Hall and Braughler, 1993; Merrill et al., 1993; Nishisho et al., 1996). Growing evidence indicates that apoptosis is an important mechanism of the death of neurons and oligodendrocytes after SCI (Crowe et al., 1997; Liu et al., 1997; Eldadah and Faden, 2000; Springer et al., 2001).

The signaling events leading to neuronal and oligodendroglial apoptosis after SCI remain to be fully delineated. In this study, SCI specifically induced DP5 expression. Bim and Bad, two other BH3-only family members, remained unchanged in the injured cord. In parallel with the time-course of the upregulation of DP5 expression, JNK and its exclusive substrate, c-Jun, were phosphorylated. Immunohistochemical studies revealed that p-JNK was expressed in neurons and oligodendrocytes (but not astrocytes) in the contused cord, and that some of these cells also expressed DP5 and the activated form of caspase 3. Basal immunoreactivity of p-JNK, DP5, and the activated form of

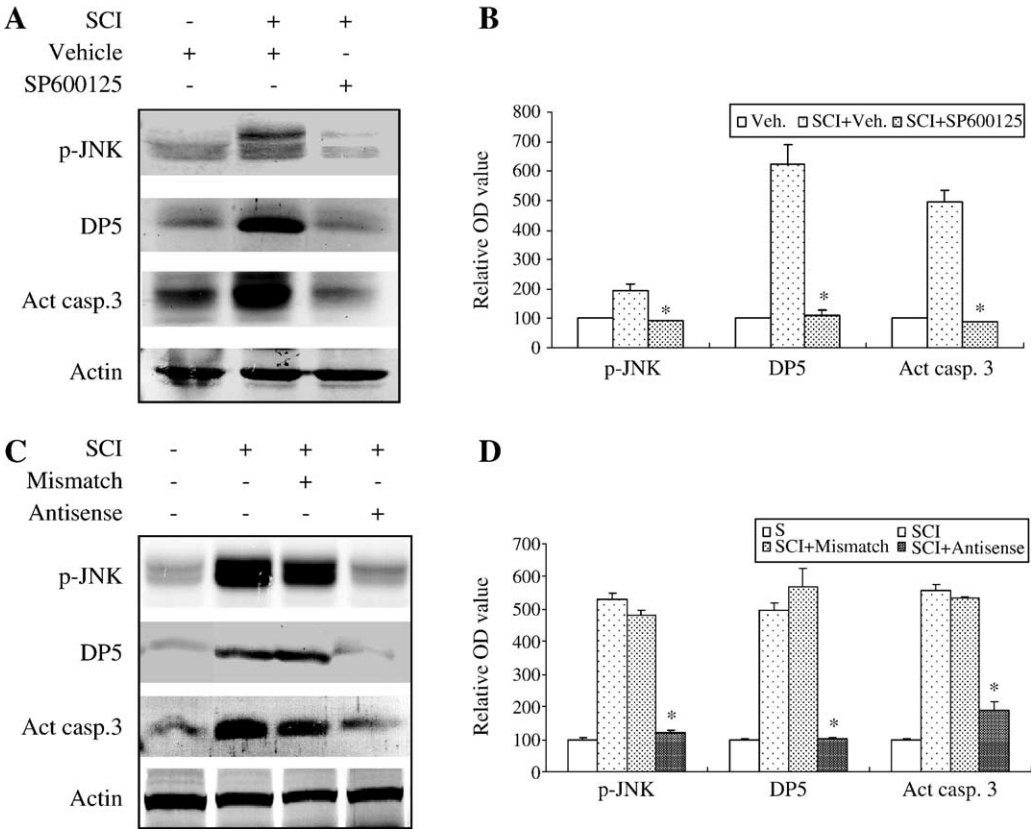

Fig. 5. Suppression of DP5 induction and caspase 3 activation by JNK inhibition after SCI: (A, B) Rats were treated with the JNK inhibitor, SP600125 (15 mg/kg s.c.) 12 h before SCI. Four h after SCI, cord segments were extracted and prepared for Western blotting, using anti-p-JNK, anti-DP5, anti-active caspase 3 (Act casp.3), and anti-actin antibodies. SP600125 significantly reduced JNK phosphorylation, decreased induction of DP5, and caspase 3 activation after SCI, compared to vehicle + SCI group. Panel A shows representative blots from 3 rats. Panel B shows data in relative density expressed as mean ± SD of 3 samples. *Denotes difference is significant from the SCI group treated with vehicle. (C, D) Rats were treated with vehicle (lipofectin), *jnk1* antisense or mismatch ODN, 16 h before SCI. Four hours after SCI, cord segments were extracted and prepared for Western blotting. Note the *jnk1* antisense ODN effectively reduced post-traumatic JNK phosphorylation compared to mismatch ODN or SCI without ODN pre-treatment. Moreover, *jnk1* gene knockdown decreased DP5 induction and caspase 3 activation following SCI. Panel C shows representative blots from 3 rats with similar results. Panel D show data in relative density expressed as mean ± SD of 3 samples. *Denotes difference is significant from the SCI group treated with the mismatch ODN.

caspase 3 was low in the uninjured cord. Suppression of JNK activity by SP600125, a JNK inhibitor, or *jnk1* knockdown by an antisense ODN attenuated SCI-induced DP5 upregulation and caspase 3 activation. These findings suggest that JNK activation mediates, at least in part, SCI-induced DP5 expression and subsequent caspase 3 activation (Fig. 6).

The JNK pathway is activated by the exposure of cells to multiple forms of stress and has been considered as a key mediator of stress-induced apoptosis (Davis, 2000). Examples include neuronal apoptosis induced by NGF withdrawal (Xia et al., 1995; Park et al., 1996; Eilers et al., 1998), excitotoxic stress (Yang et al., 1997b) and UV radiation (Tournier et al., 2000; Tournier et al., 2001), thymocyte apoptosis induced by anti-CD3 antibody (Rincon et al., 1998; Sabapathy et al., 1999) and endothelial cell apoptosis caused by diabetes-associated hyperglycemia (Ho et al., 2000). Activation of this pathway may also contribute to neuronal death in neurodegenerative diseases including Alzheimer's, Parkinson's, Huntington's Diseases and stroke (Yang et al., 1997b; Okuno et al., 2004; Gao et al., 2005). The mechanism by which JNK activation triggers apoptotic processes remains to be fully elucidated. Substrates of JNK, including the Bcl-2 family proteins, regulate cytochrome *c* release which is an important event in apoptosis secondary to mitochondrial dysfunction. Several studies indicate that the anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1 are phosphorylated by JNK in vitro and in vivo (Maundrell et al., 1997; Yamamoto et al., 1999; Inoshita et al., 2002), leading to the suppression of the anti-apoptotic activity of these proteins. However, it is questionable whether these anti-apoptotic proteins are physiological substrates of JNK (Ito et al., 1997). Another possibility is that JNK phosphorylates the transcription factor c-Jun that might in turn mediate the induction of proteins regulating cytochrome *c* release in apoptosis (Behrens et al., 1999). Indeed, JNK has been found to regulate some pro-apoptotic BH3-only proteins via transcription-dependent mechanisms (Tournier et al., 2000). Two genes in this subfamily, *dp5* and *bim*, have AP-1 binding sites on their promoters, and transcription appears to be regulated by JNK activity (Yang et al., 1997a; Davis, 2000; Harris and Johnson, 2001; Putcha et al., 2001, 2003; Whitfield et al., 2001; Yin et al., 2002). Our findings that DP5 is induced after SCI in a JNK-dependent manner are consistent with these previous in vitro reports. JNK has also been found to catalyze the phosphorylation of the BH3-only proteins, Bad, Bim and Bmf, to promote the pro-apoptotic activity of these proteins (Lei and Davis, 2003; Putcha et al., 2003; Okuno et al., 2004; Gao et al., 2005), which may also contribute to SCI-induced apoptosis. For example, a recent study by Okuno et al. has shown that JNK may directly phosphorylate Bim and thus transduces apoptotic signaling to Bax in ischemic brain injury (Okuno et al., 2004). However, we have found that SCI failed to activate 2 other BH3-only proteins, Bad and Bim. This finding is not in agreement with the previous studies. The difference in apoptotic gene expression may be due to differences in experimental models or cell death mechanisms.

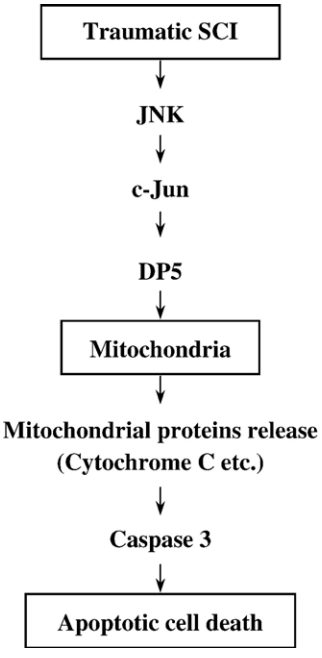

Fig. 6. JNK-dependent transcriptional regulation of dp5 expression and related events leading apoptosis after SCI: traumatic SCI results in the activation of JNK and subsequent phosphorylation of c-Jun, an exclusive substrate of this kinase. Phosphorylated c-Jun upregulates DP5 expression, initiating subsequent apoptotic cascades including mitochondrial protein release (cytochrome *c*, etc.), and caspase 3 activation.

The upregulation of dp5 gene has been shown to result in the induction of apoptosis in cerebellar granule neurons (Harris and Johnson, 2001; Harris et al., 2002), cultured rat cortical neurons (Imaizumi et al., 1999), and rat sympathetic neurons (Imaizumi et al., 1997). Inhibition of dp5 gene expression in cortical neuron may result in a blockade of amyloid beta-induced increases in cytoplasmic cytochrome *c* and caspase 3-like activity (Bozyczko-Coyne et al., 2001). In addition, increased expression of dp5 mRNA and DP5 immunoreactivity was found in the spinal neurons of patients with amyotrophic lateral sclerosis (ALS) and transgenic mouse models of ALS, implying a critical involvement of dp5 expression in ALS pathogenesis (Shinoe et al., 2001). We are the first to demonstrate that DP5 may have a role in trauma-induced apoptosis in the spinal cord, suggesting a key role in the apoptotic cascade in the injured cord. We further demonstrate that SCI-induced DP5 expression was regulated by JNK activity. The downstream events included the activation of caspase 3, an executor of apoptosis. Moreover, inhibition of JNK activity by a pharmacological or gene knockdown approach significantly attenuated SCI-induced DP5 upregulation and caspase 3 activation. Thus, we provide a link between the activation of the JNK pathway and subsequent expression of DP5, a BH3-only protein, and the activation of caspase 3. JNK inhibition has been reported to effectively prevent neuronal apoptotic death in several neurodegenerative diseases and ischemic brain damage (Yang et al., 1997a,b; Okuno et al., 2004; Gao et al., 2005). Therefore, the JNK/c-Jun/DP5/Caspase 3 signaling pathway may represents a potential target for therapeutic interventions in SCI. Generally, a useful therapy should be effective if administered after the onset of SCI. In this study, we employed SP600125, a specific JNK inhibitor, 12 h prior to injury based on two reasons. The first is that we injected this compound subcutaneously and it may take time to reach the target (the spinal cord) to exert its pharmacological effects. The second is to exert a more effective inhibition of JNK activity. Pretreatment is likely to be more effective with the notion that JNK was activated as early as 1 h post-injury. In the future, studies are needed to determine the efficacy of JNK inhibitors in post-treatment paradigms to explore their therapeutical potentials in clinical situations.

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