Sulforaphane Attenuates Matrix Metalloproteinase-9 Expression Following Spinal Cord Injury in Mice

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Abstract. Inflammation plays an important role in the pathogenesis of secondary damage after spinal cord injury (SCI). The present study explored the effect of sulforaphane (SFN), a potent anti-inflammatory extract of cruciferous vegetables, on the expression of two inflammatory mediators, metalloproteinase (MMP)-9 and TNF-α, in a murine model of SCI. Murine spinal cord injury was induced by the application of vascular clips (force of 10 g) to the dura after a three-level T8-T10 laminectomy. The wet/dry weight ratio was used to reflect the percentage of water content of impaired spinal cord tissue at 48 hr after SCI. The mRNA levels of MMP-9 were determined using the reverse-transcriptase polymerase chain reaction (RT-PCR), and protein levels of TNF-α and MMP-9 were detected by enzyme-linked immunosorbent assays (ELISA) at 24 hr after SCI. Gelatin zymography was used to determine MMP-9 activity of spinal cord tissue at 24 hr after SCI. Mice treated with SFN at 1 hr after SCI had lower expression and activity of MMP-9 compared to mice with SCI. The decrease of MMP-9 in mice treated with SFN was associated with decreased levels of spinal cord water content and TNF-α. In summary, suforaphane decreases MMP-9 and TNF-α expression and vascular permeability changes following spinal cord injury in mice.

Keywords: spinal cord injury, sulforaphane, matrix metalloproteinase-9, TNF-α

Introduction

Spinal cord injury (SCI) is a devastating pathology that may lead to permanent disability. It is generally accepted that SCI initiates a cascade of cellular and molecular events and that a combination of secondary injury factors results in progressive neuronal injury [1-3]. Among the secondary injury factors, inflammation is the major component and it plays a central role in the pathogenesis of SCI [4-6].

Functional recovery is influenced not only by the initial mechanical destruction of tissue after SCI but also by the complex secondary events that lead to early and delayed cell injury [7]. Current SCI treatments attempt to minimize the secondary injury and protect the neural elements that initially survive the mechanical injury. Furthermore, even a small gain in neuroprotection might induce functionally relevant neurologic recovery [2].

MMPs are a family of soluble and cell-surface bound zinc-dependent endopeptidases that mediate the degradation of extracellular matrix and other extracellular proteins, cellular infiltration, release of growth factors and cytokines, cell migration, tissue damage, and tissue repair [8-10]. However, excessive proteolytic activity of MMPs can be injurious, leading to numerous pathologic conditions, including inflammation and disruption of the blood-spinal barrier after SCI [11-12]. MMP-9 degrades gelatin (denatured collagens), collagen IV, V, and XI, myelin basic protein, vitronectin, elastin, and other substrates. MMP-9 is up-regulated after SCI and reaches maximum activity.

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at 12-24 hr post-injury. Neutrophils are the principal source of the MMP-9 that appears at the site of SCI, and MMP-9 mediates the TNF-α-induced increases in blood-brain barrier permeability in CNS injury [13,14]. MMP-9 plays a key role in the abnormal vascular permeability and inflammation within the first 3 days after SCI, and blockade of MMP-9 during this critical period attenuates the vascular events [15].

Sulforaphane (SFN) [1-isothiocyanato-4-(methylsulfinyl)-butane], a naturally occurring member of the isothiocyanate family of chemopreventive agents, has been shown to possess anti-inflammatory properties [16,17]. Best known for its up-regulation of several phase II detoxification enzymes, SFN inhibits TNF-α-induced NF-κB activation, which leads to reduced expression of NF-κB-regulated gene products involving MMP-9 in leukemic cells [18,19]. Therefore, it is reasonable to postulate that SFN might play a role in limiting the expression of MMP-9 in SCI. We undertook this study to assess the induction of MMP-9 and TNF-α in spinal cord after SCI, and to evaluate the anti-inflammatory effect of SFN in SCI.

Materials and Methods

Animals. The procedures conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA) and were approved by the Animal Care and Use Committee of Nanjing University. Male ICR mice (28 - 32 g body wt) were housed at 23 ± 1°C in a humidity-controlled vivarium with a 12 hr light/dark cycle, and they were given ad libitum access to food and water.

Experiment protocol. The mice were separated into 4 groups: (a) sham controls; (b) SCI; (c) SCI + vehicle (corn oil); and (d) SCI + SFN. The mice of the sham and injured groups were subjected to laminectomy alone or to experimental SCI, respectively; (n = 24 mice/group).

The mouse compression model of SCI was described previously to study spinal cord injury in mice [20-22]. Briefly, the animals were anesthetized with sodium pentobarbital (50 mg/kg, ip), and were transcardially perfused with cold saline (4°C). At 24 hr following sham operation or SCI, 18 mice in each group were sacrificed for collection of spinal cord segments for assays of TNF-α and MMP-9. Briefly, mice were deeply anaesthetized with pentobarbital sodium (80 mg/kg, ip), and were transcardially perfused with cold saline (4°C). Then the tissue segments containing the lesion (1 cm on each side of the lesion) were rapidly removed and stored in liquid nitrogen immediately. The other 6 mice of each group were sacrificed at 48 hr after operation for assays of the water content of spinal cord segments.

Spinal cord water content. The spinal cord water content was measured as previously described [27]. At 48 hr after SCI, spinal cords were removed from T7–T11 vertebral body levels, weighed, heated at 98°C for 48 hr, and re-weighted. The percent water content was calculated as [(wet weight – dry weight)/wet weight]×100.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from mouse spinal cord segments with EZgene Tissue RNA MiniPrep Kit (Biomiga, Inc., San Diego, CA) according to the manufacturer’s instructions. The cDNA was synthesized using Reverse Transcription System (Promega Corp., Madison, WI) and oligo-dT from 2 μg of total RNA. The reaction was carried out at 42°C for 50 min, 95°C for 5 min, and 5°C for 5 min. The resulting cDNA was amplified by PCR using the following primer sets: MMP-9: 5'-CTACTCTGAAGACTTGCCG-3' and 5'-CCATACAGTTTATCCTGGTC-3'; β-actin: 5'-AGTGAGCCTTGAATCTCGTA-3' and 5'-GCCAGAGCAGTAATCTCCTTCT-3'.

The cDNA was amplified by 35 cycles with a three-step temperature cycle: denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min [28]. PCR products were detected by agarose gel electrophoresis in 2% agarose gels, and visualized by ethidium bromide staining. The intensity of the bands was quantified using the ImageJ program, and the ratios of each gene product to the β-actin product were used as indices of MMP-9 mRNA expression.

Enzyme-linked immunosorbent assay (ELISA). Portions of spinal cord tissues, collected at 24 hr after SCI, were homogenized as previously described in buffer containing 1 mmol/L of phenylmethylsulfonyl fluoride (PMSF, Sigma, St Louis, MO), 1 mg/L peptatin A, 1 mg/L aprotinin, 1 mg/L leupeptin, and phosphate-buffered saline solution (pH 7.2),...
and centrifuged at 12,000 ×g for 20 min at 4°C. The supernatant was collected and total protein was determined using the Bradford method. Spinal cord levels of cytokine TNF-α and MMP-9 protein were quantified using ELISA kits specific for mouse according to the manufacturers’ instructions (TNF-α from Diaclone Research, France; MMP-9 from Biosource Europe SA, Belgium) and a previous study in our laboratory [29]. Cytokine TNF-α content in the spinal cord samples was expressed as pg/mg of total protein, and MMP-9 content was expressed as ng/mg of total protein.

**Gelatin zymography.** Samples of spinal cord, prepared from the epicenter, were quick-frozen at -80°C. Each sample was weighed and homogenized (1:4 w/v) in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM CaCl₂, 0.2 mM NaN₃, 0.01% Triton, and then mixed with electrophoresis loading buffer. Soluble and insoluble extracts were separated by centrifugation and stored at -20°C. Equal amounts of the supernatant were analyzed by gel zymography as described previously on 10% SDS-polyacrylamide gels copolymerized with substrate (1 mg/ml gelatin) [30]. The proteins were renatured by incubation in 2.5% Triton X-100 and then incubated in substrate buffer (50 mM Tris-HCl, pH 8.5, 5 mM CaCl₂) for 24-36 hr at 37°C to enable the MMP-9 to cleave the gelatin. After rinsing in water, each gel was stained with Coomassie blue for 4 hr and destained in 50% methanol. Proteolytic activities were detected by clear bands indicating the lysis of the substrate. Quantification of MMP-9 band density was performed with image analysis program ImageJ.

**Statistics.** Software SPSS 13.0 was used for statistical analyses. Data were presented as mean ± SE. The results were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons; p < 0.05 was deemed significant.

**Results**

The wet/dry weight ratio, which reflects the percentage of water content, is an index of tissue microvascular permeability. We examined the influence of SFN on the spinal cord water content of mice at 48 hr after SCI. A significant increase in the spinal cord segment wet/dry weight ratio was observed in SCI mice when compared with sham-operated mice (p < 0.01), while significant reduction was observed in SFN-treated mice when compared with SCI mice (p < 0.01) (Fig. 1).

We analyzed the spinal cord segment levels of TNF-α by ELISA in order to test whether SFN may regulate the secretion of pro-inflammatory cytokines after SCI. A substantial increase in TNF-α production was observed in spinal cord tissue samples collected from SCI mice at 24 hr after SCI (p <0.01), while spinal cord levels of TNF-α were significantly attenuated by the ip injection of SFN (p <0.05) (Fig. 2).

To assess whether SFN may modulate the expression and activity of MMP-9 after SCI, we analyzed the spinal cord tissue levels of MMP-9 by RT-PCR and ELISA and we investigated MMP-9 activity by gelatin zymography. RT-PCR for MMP-9 showed a basal mRNA level of MMP-9 in tissues from the sham mice, and a significant upregulation of MMP-9 mRNA was detected in the cord tissues from SCI-operated mice when compared with sham mice (p <0.01).

Ip injection of SFN significantly prevented SCI-induced up-regulation of MMP-9 mRNA (p <0.01) (Fig. 3). Similarly, the ELISA level of MMP-9 expression was increased at 24 hr after SCI (p <0.01), and SFN reduced the MMP-9 expression (p <0.01) (Fig. 4). Gelatin zymography showed that MMP-9 activity of spinal cord tissue after SCI was down-regulated after SFN treatment (Fig. 5).

**Discussion**

This study revealed that ip injection of SFN reduced edema and TNF-α expression of spinal cord tissue after SCI. It exerted protective effects by reducing SCI-induced MMP-9 activity and expression. Based on these results, it appears that, in mice after SCI, SFN has an important role in inhibiting inflammatory cytokines and reducing spinal cord microvascular permeability.

The primary injury of SCI is irreversible. Further progressive destruction of the tissue surrounding the necrotic core is known as secondary injury. It is delayed and therefore amenable to intervention [12,31]. Several mechanisms contribute to this pattern of destruction including inflammatory response, oxidative stress, glutamate excitotoxicity, and Ca²⁺ overload. Inflammatory response may play an important role in the secondary injury mechanism after SCI. TNF-α is considered to be a proinflammatory cytotoxic cytokine and is primarily responsible for initiating the cascade of other cytokines in the immune response [32,33]. Evidence has been reported that TNF-α induces the production of MMP-9 [34,35]. MMP-9 is produced by neutrophils and endothelia, facilitates
Fig. 1. The wet/dry weight ratio of spinal cord tissues at 48 hr after SCI. The figure shows an increase in the spinal cord wet/dry weight ratio of SCI mice compared to sham-operated mice, while a reduction was observed in SFN-treated mice compared to SCI mice (data are mean ± SE). *p < 0.01 between SHAM and SCI. **p <0.01 between SCI+SFN and SCI.

Fig. 2. The ELISA level of TNF-α in spinal cord tissues at 24 hr after SCI. The figure shows that the ELISA level of TNF-α expression was increased at 24 hr after SCI, while administration of SFN reduced the TNF-α expression (data are mean ± SE). *p <0.01 between SHAM and SCI. **p <0.05 between SCI+SFN and SCI.

Fig. 3. Differential mRNA expression levels of MMP-9 in spinal cord tissues at 24 hr after SCI. Left panel: A representative agarose gel image for MMP-9 gene product is shown. Right panel: The figure shows that the mRNA expression level of MMP-9 was increased at 24 hr after SCI, while administration of SFN reduced the MMP-9 mRNA expression (data are mean ± SE). *p <0.01 between SHAM and SCI. **p <0.01 between SCI+SFN and SCI.

Fig. 4. The ELISA level of MMP-9 expression in spinal cord tissues at 24 hr after SCI. The figure shows that the ELISA level of MMP-9 expression was increased at 24 hr after SCI, while administration of SFN reduced the MMP-9 expression (data are mean ± SE). *p <0.01 between SHAM and SCI. **p <0.01 between SCI+SFN and SCI.
leukocyte diapedesis, and may be a vascular permeabilizing factor. Consistent with other studies, we found that TNF-α and MMP-9 are up-regulated during SCI, and that spinal cord water content at the injury site is increased at 2 days after SCI [7,27].

As an isothiocyanate present in cruciferous vegetables such as broccoli, sulforaphane (SFN) has been extensively investigated in regard to its ability to induce phase II detoxification enzymes [36,37]. It activates Nrf2 and exerts anti-inflammatory effects [38,39]. Moon et al [40] reported that SFN non-specifically inhibits TNF-α-induced NF-κB activation with suppression of NF-κB-dependent genes including MMP-9 in TNF-α-resistant leukemia cells. In the present study, ip injection of SFN at 1 hr after SCI in mice attenuated spinal cord tissue edema, decreased the expression and activity of MMP-9, and inhibited inflammatory cytokine TNF-α when compared with SCI mice.

Inflammation is appropriately considered as a “dual-edged sword.” Some of the inflammatory elements, such as TNF-α, macrophages, and nitric oxide, have both neuroprotective and neurotoxic effects after SCI. There is increasing consensus that the early phases of inflammation are deleterious, whereas the later inflammatory events are protective [2]. Thus, depression of inflammation may benefit functional recovery of SCI.

The dosage of SFN (5 mg/kg) used in the present study was based on the efficacy of SFN in traumatic brain injury models in rats [24], and an ip dose of 0.5 mg/mouse effectively increased the expression of target genes in the retina of mice [25]. The initiation of inflammation after SCI of mice is quite early, so we administered SFN just 1 hr after SCI. To our knowledge, this is the first time that SFN was tested as therapy of SCI in vivo. The next challenge is to determine the relevant concentrations of SFN in the diet, bioavailability, genetic polymorphisms, toxicological testing, and whether SFN maintains chemopreventive activities through the same molecular mechanisms in clinical use.

Although the precise mechanisms underlying the interactions of SFN with inflammatory cytokines and their mediators are still unclear, there is evidence that SFN may function as a direct and also an indirect antioxidant to inhibit ROS generation and to scavenge ROS after production [25]. SFN was traditionally thought to protect cells through Nrf2-mediated induction of phase 2 detoxification enzymes that elevate cell defenses against oxidative damage [38]. Through these antioxidant activities, SFN may inhibit NF-κB activation and reduce the expression of NF-κB-regulated genes [39].

Kwon et al [41] reviewed the available preclinical research on neuroprotective therapies for acute SCI in vivo, especially those with relatively
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high potential, due to the fact that they are already used in human clinical applications, or are available in forms that could be administered to humans. These therapies include erythropoietin, minocycline, progesterone, estrogen, magnesium, and atorvastatin. Bottai et al [42] found that embryonic stem cells promote motor recovery and affect inflammatory cell infiltration in spinal cord injured mice. In this study, we found that sulforaphane decreases MMP-9, TNF-α expression, and vascular permeability changes following spinal cord injury in mice. It is difficult to compare these studies to determine which agents have the greatest protective effect, because of the variability in the design and conduct of these studies [41]. The temporal window of efficacy during which a drug might be effective in human SCI is not currently understood. Therefore, further studies and clinical trials of SFN in SCI are needed.

In summary, the present study shows that SFN plays a protective role in inhibiting microvascular leakage, inflammatory cytokine expression, and MMP-9 expression in spinal cord tissue of mice with SCI. These findings raise the possibility that SFN might be useful for treatment after SCI.

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