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Molecular insights of the injured lesions of rat spinal cords: Inflammation, apoptosis, and cell survival

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Abstract

Spinal cord injury (SCI) is a devastating neurologic injury with functional deficits. In the acute phase, which starts at the moment of the injury and extends over the first few days, numerous pathological processes begin. In this study, we made several additional advances to broaden our understanding of SCI-induced gene expression changes. We examined changes at multiple time points: 0, 6, 24, 48, and 72 h after injury, with the latter time period being added. Also, we utilized multiple analysis methods such as real-time RT-PCR, Western blot, and immunohistochemistry to increase confidence in our candidate gene and molecular processes. From the pool of information, we generated profiles of expression changes and molecular mechanisms of several injury processing. Early stages after the injury are characterized by the strong upregulation of genes involved in transcription, inflammation, and signaling proteins, and a general downregulation of neural function-related genes. In addition, edema of the spinal cord develops, and metabolic disturbances involving intraneuronal Ca^{2+} accumulation occur. This translates into a general failure of normal neural functions and a stage of signal shock that lasts for a few days in experimental rat models. Traumatic injury to the spinal cord also leads to a strong inflammatory response with the recruitment of peripherally derived immature cells, such as ED1-positive macrophages. After the trauma, apoptotic cell death continues, and scarring and demyelination accompany Wallerian degeneration. Strong expression of transcription factors of the Janus-activated kinase (JAK) and signal transducer and activator of transcription (STAT) family represents an early attempt of spinal cord repair and regeneration. Our study allowed us to conclude that combined therapeutic strategies for enhanced recovery should be performed until the chronic phase of the injury in areas distal to the lesion epicenter of spinal cords.
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Traumatic spinal cord injury (SCI) causes permanent neurological deficits because of the loss of spinal cord neurons and axons. Landmark experiments over 20 years ago demonstrated that neurological deficits are not due to an intrinsic inability of CNS neurons to regenerate, but rather to the unfavorable CNS environment [1]. The pathological changes following contusive SCI have been thoroughly documented. Axonal loss, readily apparent within 48 h after injury [2], creates a fluid-filled cyst that persists in the chronic state, with a slim rim of spared tissue surrounding the cavity. Cavitation is also present rostral and caudal to the injury epicenter. Neuronal and glial cell loss is apparent in the lesion epicenter, and rostral and caudal to the injury epicenter within 4 h of injury [3]. The devastating neurological consequences of spinal cord injuries in adults are largely attributed to retrograde neuronal cell death and failure of surviving neurons to regenerate their several axons [4]. Several approaches based on neutralizing or replacing the adverse environment in the CNS and on stimulating a robust cell body response are being explored actively in an effort to promote CNS regeneration. There are three phases of SCI response that occur after the injury: the acute phase, a phase of secondary tissue loss, and the chronic phase [5]. In the acute phase,

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which starts at the moment of the injury and extends over the first few days, numerous pathological processes begin. Mechanical lesions induce immediate change to neuronal tracks at the moment of impact. In addition, the blood flow is reduced, creating substantial ischemic necrosis [6]. This translates into a general failure of normal neural function and a stage of spinal shock that lasts for a few days in experimental animals and a few weeks in humans. In the phase of secondary tissue damage, increased production of free radicals, excessive release of excitatory neurotransmitters, and inflammatory reactions occur. Oxidative stress plays a large role in the pathophysiology of SCI [7], and contributing factors are involved: ischemia/reperfusion injury [8], generation of free radicals by activated microglia during phagocytosis [9]. Extending from days to years after the trauma, apoptotic cell death continues and, scarring and demyelination accompany Wallerian degeneration. All of these processes contribute to conduction deficits [10]. Although extensive work has been carried out to better understand the pathophysiology of SCI, our understanding of the extensive secondary injury processes is still sparse. Considering the large number of biochemical cascades and cellular reactions, a broad analysis is necessary to elucidate the phenomena involved. Many studies have investigated changes at the RNA and protein levels after spinal cord injury, but these studies have been of limited scope, focusing only on a few genes of interest [11–13]. Although these findings are significant, a more complete understanding of interactions of multiple factor, that are involved in the overall balance of promotion or inhibition is needed. It may result in greater regeneration or loss of function. The introduction of microarray technology, where thousands of genes can be studied, permits a broad assessment of gene expression changes after injury. In the chronic phase, recent advances in genomics have rendered such approaches possible. This study was performed on changes at multiple time points beyond 72 h post-injury. Furthermore, rather than merely generating gene lists, temporal and functional clustering produced comprehensive profiles of differentially expressed genes. From the pool of information we generated profiles of expression changes and molecular mechanisms of injury processing. We also verified potential signaling pathway related to cell death, survival, and proliferation after accidents. We utilized multiple analysis methods (further explained in Materials and methods) to increase confidence in our candidate signaling pathway based on expressing genes' profile. We made several additional advances to broaden our understanding of SCI-induced gene expression changes.

In the present paper, we summarized the new insights into SCI gained by use of DNA microarrays and discuss whether this new approach can obtain valuable information related to cell death and regeneration signaling in the search for new therapeutic strategies in this field. These results can be valuable for fully understanding of microenvironment signal mechanisms and the definitive roles of differentially expressed physiological factors in traumatic injured spinal cord tissues. We also discuss the potential influence of these changes on major post-injury events such as apoptosis, inflammation, synaptic plasticity, and regeneration. Ultimately, the long-term goal of future studies is to establish protocols that can be used to modulate or control pathological microenvironment signaling pathway and will certainly yield new therapeutic information along with novel treatment modalities of spinal cord injury.

Materials and methods

Experimental design. Five different post-operative time points (0, 6, 12, 24, 48, and 72 h after the injury) of spinal cord were examined in this study. At each case, three rats with laminectomy and contusion conditions were utilized for our experiments. Three separate tissue samples were then taken from each animal as follows: A, B, C; 5 mm segment encompassing the injury epicenter was taken. The epicenter sample contained not only the center of the lesion (where the damage was most severe) but also the entire lesion area, including inflammatory cells that invaded the injured tissue. Because contusion lesions enlarge over time [3], in animals that died hours after the injury, the 5 mm epicenter sample also contained undamaged tissue at either side of the lesion; by contrast, in animals that died days after injury, the lesion comprised areas of secondary damage that extended throughout the epicenter sample.

Animals and spinal cord injury (SCI) induction. Adult female Wistar rats weighting 200 g or 5-week-old rats were used in our experiments. Experimental animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Korean regulations on protection of animals used for experimental and other scientific purposes. Animals were subjected to a traumatic injury followed by modified protocol as described in detail in Honmou et al. [16]. Briefly, rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg). The spinous processes of T9 and T10 were partially transected with rongeurs to expose the dorsal spinal cord. The dura was incised with microscissors. The dorsal and ventral columns, which include the dorsal and ventral corticospinal tract (CST), were partially cut by lowering the microscissors attached to a stereotaxic arm to a depth of 3.5 mm below the dorsal surface of the spinal cord. For this study, we collected enough tissue which was harvested in a timely way for biochemical examination.

RNA extraction and semi-quantitative RT-PCR (reverse transcription polymerase chain reaction). Total cellular RNA was extracted with Trizol (Life Technologies, Frederick, MA), reverse transcribed into first strand cDNA using an oligo(dT) primer amplified by 35 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min) of PCR using 20 pM of specific primers. PCR amplification was performed using the primer sets. All primer sequences were determined using established rat GenBank sequences for genes indicative of neural lineages or control genes. Duplicate PCRs were amplified using primer designed β-actin as a control for assessing PCR efficiency and for subsequent analysis by 1.5% agarose gel electrophoresis. For labeling of PCR products, we used a Syber green detection kit which was purchased from Applied Biosystems (Foster City, CA). Twelve genes were selected for further quantitative analysis by real-time RT-PCR using an ABI7700 Prism Sequence Detection System. Primer sequences were designed using Primer Express software (PE-Applied Biosystems, Warrington, UK) and gene sequences obtained from the GenBank database. For Microarray analysis, total RNA was isolated from normal spinal cord tissue and an injured spinal cord tissue section (T9–T10). RNA isolation and purification was performed using a Qiagen RNeasy kit under RNase-free conditions (Qiagen, Valencia, CA). Purity was determined by A_{260}/A_{280} and a negative result after PCR for genomic contamination. Specificity of cDNA microarray was confirmed by randomly selecting 19 of the genes and proteins for assessment by semi-quantitative RT-PCR and Western blotting. We performed these experiments in triplicate.

Oligonucleotide microarray and data analysis. Normal spinal cord tissue and injured spinal cord tissues (0, 24, 48, and 72 h after injury) were harvested and total RNA was isolated for microarray analysis. RNA

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