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The Differentiation of Phase of Spinal Cord Injury Based on the Changes in Gene Expression

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Study Design: An experimental study.
Objectives: To define the phases of chronic spinal cord injury by researching the changes in gene expression.
Summary of the Literature Review: The exact time of conversion from acute stage to chronic stage in spinal cord injury is unknown.
Materials and Methods: We used 18 month-old Beagle dogs as study subjects. Under spinal cord monitoring, we underwent laminectomy on thoracic vertebra 10 and 11, and induced cord injury by a weight-drop injury method. Dogs in each group with spinal cord injury and group without spinal cord injury on POD 1, 7, 30, and 90. The motor functions were evaluated using the Tarlov scale. Tissues were prepared from 0.5cm up and down from the 10th thoracic level. Additional cephalic and caudal lesions from the injured site were prepared. We have checked the differentially expressed gene(DEG).
Results: The mean Tarlov value was 0.67 which indicated a significant cord injury. 4 DEG (GP3, 9, 25, 34) were detected among 40 primers after screening, the detection percentage of which was 10. In the tissues of study subjects with spinal injury, DEG was found at the injury site and cephalic lesion. DEG expressed GP3, GP9 and GP34 started expression on day 30, and GP25 was expressed on day 90.
Conclusions: According to the changes in gene expression, the day 30 would be considered as the date of conversion from acute to chronic phase of cord injury. Inhibiting secondary inflammatory change and apoptosis following spinal cord injury until this period would maximize the effect of chronic phase therapy such as cell-transplantation.

Key Words: Spinal cord injury, Differentially expressed gene

INTRODUCTION

Spinal damage occurs when the spinal cord's change in position, caused by external trauma, compresses the spinal cord. Along with a mechanical primary damage, compression of the spinal cord brings about a localized change in the blood flow that causes ischemia and edema, and causes petechae thereby further damaging the spinal cord. Following these acute pathological changes, the activation and proliferation of various cells occur after the damage over a few days. Representative of these are hyertrophy and proliferation of astrocytes, and these reactive protoplasmic astrocytes cells are important causes of cicatrizations proximal to lesions; activated astrocytes and macrophages inhibit regeneration of axons by producing extracellular matrix derived protein that interferes with the growth of axons.[1,2] Among the observed changes during the chronic phase of a spinal cord damage, the important phenomena are the continuing demyelination and apoptosis as pathogenesis. The delayed necrosis of the oligodendrocyte, which is essential for nerve regeneration, occurs a few weeks after the damage; in the recent studies, it was made known that necroses of demyelination and oligodendrocyte can continue to occur even after a significant amount of time has elapsed, with the discovery of demyelination occurring gradually for over a

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year.[3] As such, it can be thought that, after a cell transplantation, phenomenon such as demyelination must be prevented to foster restoration of nerve functions.

The author of this study believes that how to view the transition point of spinal damage-to-chronic stage is a very important issue in selecting from the various aforementioned treatment methods. Since the cell activations for the acute stage and chronic stage are different and because of the fact that even in the chronic stage various cell activations continue, the author's questions are thought to be worthwhile, therefore, this study was conducted. Presuming that the point at which genes show significant changes is the chronic stage for a spinal cord damage, if which genes show changes after the damage is made known, it was assumed then that it would be possible to know the treatment point and duration for the acute stage treatment, the appropriate timing for gene therapy such as cell transplantation, and the treatment target genes.

RESEARCH SUBJECTS AND METHODS

1. Test Animals

Male beagle dogs with the average age of 18-months and average weight of 5.8 kg (3.7 kg - 7.2 kg) were used. After using intravenous injection of ketamine to induce anesthesia, a breathing tube was inserted and propofol (Baxter) was used to maintain anesthesia by continuous infusion. In order to verify the exact extent of spinal cord injury not including any damage that occurred during the operation, spinal cord monitoring was conducted. For the monitoring, a NIM-SPINETM (Medtronic, Sofamor Danek, Memphis, TN, USA) instrument capable of transcranial electrical stimulation - motor evoked potential continuous and electromyography monitoring was used. For percutaneous electrical stimulation, two electrodes were inserted into the scalp and recording electrodes were inserted into the left, right, top bottom locations above the motor nerves cord surrounding the surgical site. After the sterile manipulation of the entire thoracolumbar, incisions were made in the skin and muscles and conducted laminectomy proximal to the thoracic vertebrae 10 and 11 without damaging the dura mater. After laminectomy spinal cord damage was induced using a weight-drop injury method (20 cm / 5 g) with 3 mm diameter rods. During this, spinal cord damage was verified with biphasic wave forms from the EMG and motor-evoked potential tests. There were 10 test animals; although on the 1-day, 7-day, 30-day and 70-day marks the same operation was performed on the test animals with spinal cord damage and the control group animals according to the test plan, the test animals without spinal cord damage were sacrificed 1 at a time, and, since 2 test animals died during testing (1 from the test group and 1 from the control group), a total of 8 test animals were used for this study. To prevent urinary tract infections and to maintain the artificial urinary bladder function artificial urination (twice a day) was conducted, and for 3 days after the surgery cefazoline (0.5 mg/kg) was administered.

Table 1. Modified Tarlov's scale for motor function evaluation

Scale	Degree of Paraplegia	Symptoms
0	Flaccid	Minimal or no leg movements; variable tone; loss of bowel/bladder control
1	Spastic	Moderate or vigorous purposeless leg movements; leg spastic and extended; no sitting; loss of bowel/bladder control
2	Severe	Only moderate spasticity in legs; vigorous, coordinated movements suggesting walking; can sit and stand; loss of bowel/bladder control
3	Moderate	Sitting, standing, and walking are shown; legs and hips obviously unstable; leg lagging; loss of bowel/bladder control
4	Mild	Walking with some leg and hip instability; bowel/bladder functional
5	Normal	Hip instability seen only with jumping or running

2. Measurement of Movement Difficulties

Behavioral recovery was measured using the Modified Tarlov Scale after anesthetic recovery; the spinal cord damage was verified by evaluating the functionality of the lower limbs (Table 1).

3. Preparation of Specimen

After the spinal cord damage, on the 7-day, 30-day, and 90-day marks the test animals were sacrificed; using 4% paraformaldehyde, perfusion fixation was conducted; the test specimens were cut in cross sections including 0.5 cm of top and bottom of the damaged parts (T10), and the head part and tail part of the damaged area were cut and they were fast-frozen using liquid nitrogen and the total RNA were extracted using TRIzol.

4. Polymerase Chain Reaction (PCR)

(1) Denaturation

10 μg of RNA, 10 μl of RNase inhibitor (1 U/μl), 1 μl of RNase-free DNaseI (10 U/μl), 5 μl of 0.1 M Tris-Cl pH 8.3, 5 μl of 0.5 M KCl, and 5 μl of 15 mM MgCl2 were mixed for 30 minutes at 37 °C and incubated, and afterwards a round of phenol was extracted. 5 μl of Na-Acetate and 200 μl of 100% Ethanol were precipitated and incubated for at least 30 minutes at -80 °C. After rotation and cleaning were done, it was dissolved in 20 μl of H2ODEPC; RNA concentration was measured, and the conservation condition of the denatured gel was examined.

(2) Synthesis of cDNA

For a single RNA, 4 reactions were set by putting it into test tubes each filled with one of the following degenerate anchored oligos (dT) primer sets: T12MA, T12MC, T12MG, or T12MT. DNA-free RNA was diluted with 0.1 μg/μl H2ODEPC and, while maintaining in frozen state, its cDNA synthesis with each of the degenerate anchored oligos (dT) were set.

(3) Polymerase Chain Reaction (PCR)

For all the PCR reactions that have the same T12MN primer, a mastermix was prepared and 18 μl of it was added to each test tube, and afterwards arbitrary primer (= decamer) was added to each test tube. For each of the 20 μl of PCR reaction, 9.2 μl H2O, 2 μl 10 x PCR reaction buffer, 1.6 μl 4dNTP mix (25 μM), 2 μl T12MN primer, 2 μl cDNA, 0.2 μl Taq DNA polymerase (5 U / μl), 1 μl [a-33P] dCTP were mixed. After quartering the mixture, 2 μl of arbitrary primer (2 pmol/μl) was added. The PCR was manipulated at 94 °C for 30 seconds, at 40 °C for 2 minutes minimum, and at 72 °C for 30 seconds for 40 cycles, and at 72 °C for 5 minutes for 1 cycle. Until it became a gel, the PCR reaction was stored at -20 °C.

(4) Termination Reaction

As soon as cover reaction was complete, 2.5 ~ 3.5 μl was added to each of the 4 test tubes and mixed well. After incubating for 5 minutes at 37 ℃, reaction was terminated by putting in 4 μl of stop solution in each of the 4 test tubes. In the prepared gel plate, shark tooth comb was inserted and reactants were added.

5. Activity Level of Luciferase

To evaluate the in vitro transfection (infection of the cells of the isolated nucleic acid), the cells were washed twice with PBS and lysed with 200 μl of soluble buffer. After 15 minutes of room temperature incubation, it is moved to ultracentrifuge and spun for 15 seconds, and centrifuged for 3 minutes at 11,000 rpm. The extracts then were transferred to new test tubes and store at -70 ℃ until use. The protein concentration of the extract was determined using a BCA protein kit. The activity level of luciferase was measured with relative light unit (RLU) that uses a light meter, and this was observed for 30 seconds. The RLU/mg total protein was used for the final value of the Luciferase.[3)]

6. Screening Standard for Differentially Expressed Gene (DEG)

In order to screen reproducible DEGs in re-testing, in this test DEGs were screened within a PCR product size range of 300~1,200 bp, and screened for only those DEGs with at least 2X the difference in expression.

7. Statistical Analysis

Student's t-test was used to compare the differences between the groups, and if less than $p < 0.05$, then this was considered statistically significant.

RESULTS

1. Kinematic Assessment

The nerve damage in all the test animals was evaluated using the Tarlov Scale, and the average value during the entire testing period was 0.67 (0.4 to 1.3) , and it showed significant nerve damage.

2. Total RNA Extraction

By correctly defining all the RNAs from the spinal cord test specimens used in each testing period, it was possible to verify that all the RNAs had been correctly defined before testing (Fig 1).

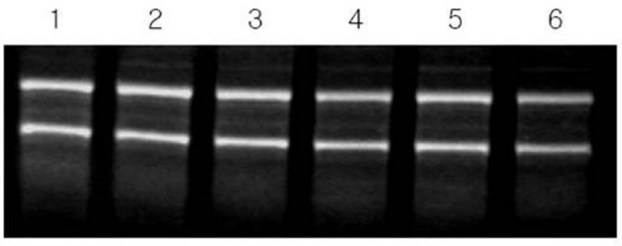

Fig. 1. The result of total RNA extraction. The exactly same amount of RNA was extracted before every experiment.

3. Activity Level of Luciferase

The changes in luciferase activity levels for each testing period, in the control group and the experimental group, were extracted on 1-day, 7-day, 30-day, and 90-day marks, and on 7-day, 30-day, and 90-day marks, respectively; the results showed statistical difference, and these differences were the largest for the control group on the 7-day mark at 0.3 RLU/mg total protein, and the largest for the experimental group on the 7-day mark at 1.7 RLU/mg total protein (Fig 2).

4. DEG

DEGs were screened according to the criteria established before testing, and anything that did not meet the criteria were eliminated.

(1) DEG Incidence and Location

In the DEG screening using a total of 40 primers, a total of 4 DEGs were expressed (a 10% expression rate) and the expression sites were GP3 (Fig. 3), GP9, GP25, and GP 34.

(2) DEG Expression Site

GP3 was expressed at the tail of the damaged spinal cord; GP9 was expressed at the area of the damaged spinal cord; GP25 and GP34 were expressed only at the damaged spinal cord and the tail of the damaged spinal cord. No expression occurred at the head of the damaged spinal cord.

(3) DEG Expression Timing

DEGs were not expressed on the 1-day mark and 7-day mark; GP3, GP9, and GP34 started expression on the 30-day mark; GP25 was expressed only on the 90-day mark.

DISCUSSION

During the acute stage of spinal cord damage, it is important to regulate or inhibit inflammatory cytokine; recently, the biomechanical pathways related to various cytokines and their receptors have been attained, and the researches concerning the directions of these pathways promoting eliminating or inhibiting cells are progressing actively.[4] In contrast, because during the chronic phase of spinal cord damage a gap develops in the spinal cord finally, instead of regulating inflammatory changes or cell elimination, the method that enables cell transplantation of the neural stem cells or progenitor cells for reducing and joining the gap is recommendable.[5,6-8] Although the mammalian target of rapamycin (mTOR), which regulates growth of nerve cells in their creation stage, becomes activated, after the nerve cells have grown the activities of the growth pathways become limited, and, afterwards, the growth pathways become completely closed off if nerve cells become damaged, and regeneration of nerve cells becomes impossible.[3,9] These explanations, if one were to think the contrary, then an assumption can be made that the growth pathways can be reactivated if the key genes that inhibit these growth pathways, e.g., PTEN and TSC1, can be stopped.[3,10] In one experiment with a group of mice that had two gene removed from their brain cells and a group of mice that had their optic nerves damaged mechanically, the group that had genes manipulated showed 50% re-growth of damaged nerve whereas the other group showed only 20% re-growth; 10% of the mice in the group with manipulated genes showed significant growth of axons. Even if the axons in the brain and spinal cord do not have regenerative abilities, the peripheral nervous system has a remarkable ability to regenerate. After the central nervous system and peripheral nervous system were damaged, the difference in regeneration results would be related to the difference in gene expressions; this difference in gene expressions explains the complexity and variety of the spinal cord physiology after the spinal damage.[11] Based on this

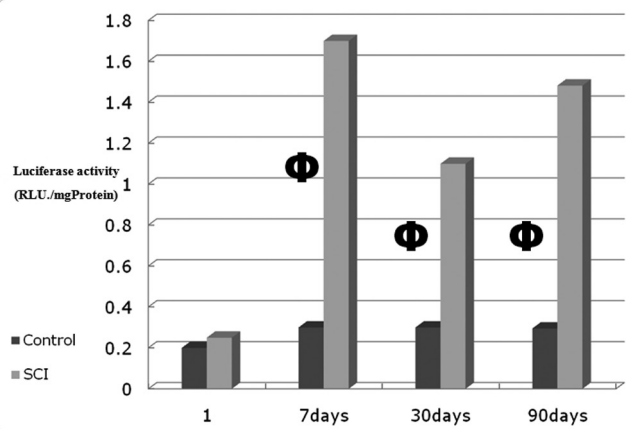

Fig. 2. The results of luciferase activity. Φ: means statistical differences.

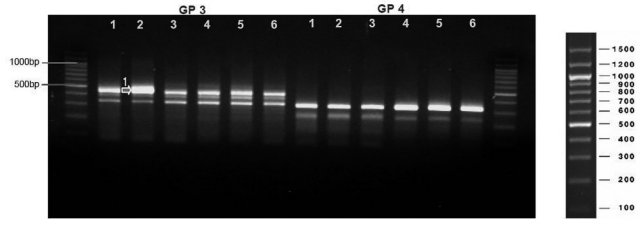

Fig. 3. Noted differentially expressed gene(DEG) at GP3. The degree of expression is more 2 times in spinal cord injured dog than control.

kind of research background, the author of this study planned experiments using dogs, since dogs are mammalians and they can provide data that are closer for gene research. In addition, in the experiments of this study it was assumed that, after spinal cord damage, the point when gene changes occur in spinal cord, is the point in time where gene changes are at maximum and spinal cord damage to be at maximum.[11,12] With this kind of assumption, and in order to prove this assumption, the author of this study wanted to compare the gene sequences of the dogs with induced spinal cord damage with those of the normal dogs; the author attempted to find out which and when gene changes start, and where in the spinal cord gene changes generally occur.

SM216289, the recently extracted small molecule compound from strains, were found to inhibit Semaphorin3A in vivo. Summarizing the study, Semaphorin3A has the ability to inhibit axon regeneration, after a spinal damage, in the areas proximal to the damage; in vitro and in vivo, it has the ability to inhibit generation and protection of axons, regeneration of myelin, angiogenesis, and cell elimination hindrance.[13] Therefore, in the currently on-going research of Yang et al.,[14] the combination of substances that regulates Semaphorin3A, as a treatment method for the nerve damage that occurs after spinal cord injury in humans, could propose a new future direction. However, how much longer administering this kind of combination therapy would continue remains as a huge challenge. In this sense, the author of this study is confident that this research will provide important guidelines for the future clinical research attempts. To confirm this sort of results, gene fishing was used, and this method is characterized as DDRT-PCR; applying DDRT-PCR for gene fishing is known as DEG-PCR. This method produces one strand of cDNA by using degenerate anchor primer; by using the same anchor primer and arbitrary decamer primer with random nucleotide sequence and by amplifying a part of the area of cDNA, as a method to verify differences between different tissues and cells by using the existence of mRNA, therefore, it was considered to be an apt research method and was used in the experiment of this study. In the experiment, by identifying the total RNA, only the cases where an equal amount of RNA was identified before testing were tested.

Bioluminescence Resonance Energy Transfer (BRET) technique is a very useful experimentation method for proteomics and functional genomics including the research area of signal transduction mechanisms in living cells.[15] Generally, the phenomenon of delivery of fluorescence energy between two kinds of fluorescent phosphors to determine the cross-coupling of proteins. However, due to the fact that it cannot be used for autofluorescence that occurs unexpectedly in other substances or for photoresponsive retina cells, and due to fluorescence compounds used as donor material becoming photobleached and unable to be used for a long period of time, the fluorescence resonance energy transfer method has limitations; BRET is an improved method that addresses these limitations.[16] Although BRET also uses the phenomenon of delivery of resonance energy between donor molecules and acceptor molecules, unlike FRET, it differs by using luminescent material instead of fluorophore emitting materials.[16] Protein containing Renilla luciferase (Rluc) gene act as donor molecules; protein containing GFP2 gene act as acceptor molecules; the excited state of donor molecules does not use the light energy used in FRET but the light-emitting energy induced by DeepBlueC that has a characteristic of not involving heat generation. In this experiment, the 7-day, 30-day and 90-day marks all showed statistical differences, and these differences showed up the largest on the 7-day mark. Interpreting, this signified that after 7-days of damage the changes in proteins occurred rapidly. But, it was unclear as to whether the change in luciferase was a true gene change or a change in inflammatory mediators or a change in various transcription factors.

In order to compare and analyze the changes in genes with normal tissues, a more defined genetic screening method was presented in this experiment. In other words, DEG was screened in the PCR product size of 300 ~ 1,200 bp band, and only the expressions greater than 2X of the band intensity were observed. The expression rate of DEG was 10%, based on a total of 40 primers in this experiment. If the number of primers were to be increased, considering the sequences of genes, there would be more DEGs expressions found. The expression sites were GP3, GP9, GP25, and GP 34. The pattern of DEG expressions was not constant. DEG expression site is important; GP3 expression took place in the tail area of the damaged spinal cord; GP9 expression took place in the area proximal to the spinal cord damage; expressions for GP25 and GP34 took place only in the area proximal to the spinal cord damage and the tail area. In other words, no expressions took place in the head area of the

spinal cord damage, and whether this was attributable to the testing period being 90-days was questionable; it is warranted that verification be done in the future with longer testing periods concerning this. DEGs were not expressed on 1-day and 7-day marks; GP3, GP9 and GP34 started expressions on the 30-day mark; GP25 expression took place only on the 90-day mark. Comparing the results of this study and the results of luciferase, the changes in luciferase that started occurring on the 7-day mark was perhaps attributable to the changes in various inflammations and related proteins. And it can be viewed that, after the 7-day mark, changes in inflammatory proteins and changes in the actual genes would accompany each other. Considering only the DEG expression timing, since the changes in some genes are verified after 30 days of spinal cord damage, the maximum period for the acute stage can be appropriately estimated as 30 days, based on the assumption of this study. Considering only the results of this study, although additional testing to see what types of genes undergo how much change may be necessary and the 90-day mark changes were verified, after 30 days it should be considered chronic stage and appropriate treatments should be selected.

The strategy of preventing demyelination by transplanting stem cells that can be differentiated into oligodendrocyte, which is very important for nerve regeneration, can improve nerve conduction through the axons not severed mainly by the damage, however, it will not have much effect on the already-severed axons.[17] The reason for that is the already-severed lower axons are disintegrated by the Wallerian degeneration, and, even if the upper axons become re-myelinized, they cannot affect the neuron signaling by the lower motor neurons because the axons are already severed.[18,19] From this perspective, the strategy of preventing demyelination by transplanting stem cells that can be differentiated into oligodendrocyte ends up limited in its effectiveness. Therefore, in order to improve the lost neurological function after spinal cord injury in a more substantive way, an accompanying strategy to promote regeneration of severed axons would need to be prepared.

In regard to the strategy of inducing axonal regeneration through stem cell transplants, one thing that needs to be aware of is the fact that uncontrolled over-expression of neurotrophic factors could bring unwanted side effects.[20,21] In reality what's been reported is that, in animals which underwent neural stem cell transplantations, the innocuous-stimuli-pain of sensing even the mildest stimuli occurred, and that these innocuous-stimuli-pains were found to be associated with excessive growth of pain-related small sensory neurons. What is interesting to note here is that, when neurogenin-2 was used to induce differentiation into neurons and then transplant was performed, the abnormal growth of the innocuous-stimuli-pain and sensory neurons had decreased.[22] These facts suggest that the more differentiated the cells, the less secretions of nonspecific nutritional factors, and, accordingly, that it is possible to reduce the side effects of the uncontrolled axonal growth. As mentioned above, using cell transplantations during the acute stage requires utmost care; in addition, if the issues that are recognized as being stem cell-associated such as transplantation of multiple cells, the maintenance and differentiation of transplanted cells, and ethical issues were to be resolved, then the chronic stage of spinal cord damage, in other words, after the 30-days after damage, staring a rehabilitative treatment for spinal cord should be considered. However, after the acute stage of spinal cord damage, within 30 days inflammation should be minimized and efforts are absolutely needed to minimize spinal cord cavitation; for this, the recent researches on antagonists for inhibiting axon regeneration should continue further. The study of this report acknowledges its limitations, which stem from the small number of objects of only 4 test animals each for the experimental group and the control group and due to the diverse DEG expressions. The future studies will require more discoveries of DEGs using primers and regulated studies of DEG.

CONCLUSION

In the experiment using normal dogs and dongs that were induced with spinal cord damage, starting after 30 days of damage significant changes in genes were observed; in the test of up to 90 days, all the changes in genes were observed only in the areas proximal to the spinal cord damage and the tail area of the spinal cord damage. Based on these results, the criterion for acute stage and chronic stage ought to be about 30 days after the day of damage, and this will result in effective treatment during the chronic stage.

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유전자 변화를 근거로 한 척수손상의 단계의 구분

이준호 • 양준영 • 이준규 • 주용범 • 차수민
충남대학교 의학전문대학원 정형외과학교실

연구 계획: 실험적연구
목적: 척수 손상 후 유전자 변화를 연구하여 척수 손상의 만성기의 정의를 내리고자 하였다.
선행문헌의 요약: 척수 손상의 급성기에서 만성기로 전환되는 시점에 대한 연구가 현재까지 알려져 있지 않다.
대상 및 방법: 평균 18개월 된 10마리의 Beagle종 개, 수컷을 사용하였다. 척수 신경 감시하(Spinal cord monitoring)에 흉추 10, 11번에서 추궁절제를 시행하였다. 추궁절제 후 Weight-drop injury 방법으로 척수 손상을 유도하였으며, 실험 계획에 따라 척수 손상후 1일, 7일, 30일, 그리고 90일에 척수 손상을 가한 동물과 대조군으로 정상 개를 각 한 마리씩 희생하였다. Tarlov scale을 이용하여 마취 회복 후 하지의 기능을 평가하여 척수 손상을 확인하였다. 척수 조직 절취는 횡단면으로 손상 부분(흉추10번)의 위 아래 척수 조직을 약 0.5 cm씩 포함하여 절취하였고 손상을 받은 부위의 두부와 미부에서도 조직을 절취하여 Differentially expressed gene(DEG)을 관찰하였다.
결과: Tarlov scale은 모든 실험 기간 내에 평균 0.67로 의미 있는 신경 손상을 보였고 총 40개의 primer를 이용한 DEG선별에서 총 4개의 DEG(GP3, 9, 25, 34)가 발현되어 10%의 발현율을 보였다. DEG는 척수 손상을 받은 개의 조직 중 손상부위와 미부 조직에서 발현되었다. DEG는 Gene Product(GP) 3, 9, 34가 30일째에서 발현이 시작되었고 GP25는 90일째에만 발현이 되었다.
결론: 척수 손상의 급성기와 만성기의 기준은 유전자 변화로 볼 때 손상일로부터 약 30일 정도가 기준이 될 수 있을 것이며, 이 기간까지는 척수 손상 후 발생되는 이차적 염증 변화와 세포사멸을 최대한 억제함으로써 만성기에 적용 가능한 세포 이식술 등의 치료 효과를 최대한으로 높일 수 있을 것으로 생각된다.

색인 단어: 척수 손상, Differentially expressed gene
약칭 제목: 척수 손상의 급성기와 만성기의 구분에 대한 연구