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Original Article

The experimental study of hypoxia-inducible factor-1α and its target genes in spinal cord injury

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Study design: Animal model of compressive spinal cord injury (SCI), reverse transcription-polymerase chain reaction (RT-PCR), *in situ* hybridization (ISH), immunohistochemistry (IHC) and enzymehistochemistry (EHC) were used to test the hypothesis that hypoxia-inducible factor-1α (*HIF-1α*) and the target genes activated by HIF-1α are involved in cell hypoxia tolerance and tissue vascularity to help injured tissue to go through the stress disease.
Objective: To determine whether *HIF-1α* and its target genes associated with hypoxia tolerance and neovascularization take part in the pathophysiological procedure of SCI in rats.
Setting: Yunnan University, China.
Methods: Random-bred adult male Sprague–Dawley (SD) rats weighing 250 ± 50 g were prepared for compressive SCI models. After receiving compressive injury at T_{10}, rats were sacrificed at different times from 6 h to 1 week after injury. The injured cords were removed, and HIF-1α and its target genes were assayed by RT-PCR, ISH, IHC and EHC. The data were statistically analyzed.
Results: An increase in *HIF-1α mRNA* expression was observed 12 h postinjury, reached a maximum at 3 days, and reduced gradually thereafter. HIF-1α protein expressed earlier than *HIF-1α mRNA*. Additionally, two glycolytic enzymes and vascular endothelial growth factor (VEGF), which are regulated by HIF-1α, also increased after an interval postinjury, and their expression patterns shared a same trend with that of HIF-1α protein.
Conclusion: The findings suggested that the most important hypoxic regulatory factor HIF-1α was upregulated in involved cells by activating the transcription and increasing protein stability, and subsequently activated the expression of HIF-1α target genes, including glycolytic enzymes and VEGF in SCI. Combined with the pathologic observation, it suggested that overexpression of *HIF-1α* and its target genes might take part in hypoxia tolerance and vascularity of the injured spinal cord.
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Keywords: acute spinal cord injury; ischemia; *HIF-1α*; target genes; expression pattern

Introduction

Acute spinal cord injury (SCI) is characterized by its progressive nature, which cannot be entirely explained by the primary mechanical trauma. A series of secondary injuries including ischemia, vascular changes, electrolyte disorders, edema and loss of energy metabolism, which can significantly increase the severity of SCI, have been observed in injured and adjacent segments after the acute postinjury phase.[1] Among all of secondary injuries, ischemia has been demonstrated as a focus of postinjury pathophysiological changes of acute SCI since it was believed to aggravate other secondary injuries and is parallel to the neurological dysfunction. Now, more and more studies on postinjury ischemia are paying attention to the adaptive responses of tissues or cells and their molecular mechanism. With the understanding of the pathophysiological background of ischemia, it is possible to establish specific therapeutic strategies to elevate the outcome of SCI patients.

In the past decade, it has been shown that a number of genes are regulated under oxygen tension to help cells or tissues adapt to adverse environment, among which hypoxia-inducible factor-1α (HIF-1α), an important transcriptional factor activating the transcription of erythropoietin (EPO), was firstly identified in hepatoma

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cell line.[2] This trans-acting factor has been revealed to be widely expressed in ischemic and tumor tissues.[3–5] As a member of HLA/PAS protein family,[6] HIF-1α could bind to the corresponding DNA motif heterodimerically and activate its target genes' transcription in hypoxic environment, such as inducible nitric oxide synthase (*iNOS*),[7] glycolytic enzymes,[8] vascular endothelial growth factor (*VEGF*)[9] and glucose transporter-1 (*GLUT-1*),[10] which participate in cell adaptation to hypoxia by means of the metabolic changes or/and the remodeling of microcirculation. Furthermore, some reports on the relationship between HIF-1α and the central nervous system (CNS) showed the activation of HIF-1α in the hypoxia-induced ischemic tolerance following brain injury, and revealed that it contributed to protective brain preconditioning.[11–14] For example, preconditioning with cobalt chloride ($CoCl_2$) or desferrioxamine (DFX), the known HIF-1 inducers, before hypoxia–ischemia, afforded 75 and 56% brain protection, respectively.[12] Even though the spinal cord undergoes ischemia and subsequent hypoxia in acute postinjury phase in SCI, the expression of HIF-1α has not been well documented.[15]

In the present study, a compressive rodent model of SCI was established. The expression patterns of HIF-1α in injured spinal cord were studied at mRNA and protein level, and some important target genes of HIF-1α that participated in hypoxia tolerance and vascularity of tissues were also studied to explore the relationship between expressional network of HIF-1α and development of SCI.

Materials and methods

Animals

The animals were given food and water freely before and after surgery. All experimental protocols pertinent to animals were given prior approval by the Laboratory Animal Care and Use Committee of the medical school. We minimized the number of animals used, and used anesthesia appropriately to minimize their suffering.

A total of 35 random-bred adult male SD rats weighing 250 ± 50 g were anesthetized with 5% chloral hydrate (6 ml/kg). SCI was induced based on the modified method of Taoka.[16] A laminectomy was performed centered to T_{10} segment and the lamina was ground into a 5-mm-wide, 12-mm-length window, while the dura matter was kept intact. With a compressive facility, the exposed spinal cord (spinal cord level T_{10}–T_{11}) was compressed by a 30 g weight for 10 min. Hemostasis was secured and the wound was rinsed with sterile saline. The muscles and skin were sutured with 4-0 silk. This disturbance resulted in immediate and complete hindlimb neurologic deficit. Each rat was housed individually and its bladder was emptied by manual compression twice a day until automatic bladder function recovered. Rats were randomly assigned to each of the following postoperative time intervals ($n = 5$ in each group): 6 h, 12 h, 1 day, 2 days, 3 days, 1 week, and a group with laminectomy was taken as a control. The spinal cords from 2 mm rostral to 2 mm caudal to the center of injury were sampled and these specimens from rats would be processed for regular histological evaluation, immunohistochemistry (ISH), immunohistochemistry (IHC) and enzymehistochemistry (EHC) as well.

Semiquantitative reverse transcription-polymerase chain reaction

The total RNA was isolated using TRIZOL reagent (Invigrogen) following manufacturer's instructions. RNA concentration was determined by measuring absorbance at 260 nm using a UV spectrophotometer. Primers for internal control *β-actin* were employed and resulted in a 956 bp product. Primers for *HIF-1α* (sense: 5′TGAACAGGATGGAATGGAGCA3′, antisense: 5′TGTGATCTGGCATTCGTAAGG3′) give rise to a 443 bp product. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with RT-PCR kit (Promega) following the manufacturer's instructions. After 25 cycles of reaction, the PCR products were separated by agarose gel electrophoresis. The gel was stained with ethidium bromide (EB) and PCR products were visualized by image analysis software (Molecular Analyst). The mean optical density (OD) of the PCR products of *HIF-1α* and *β-actin* was quantified with BioMias2000. The ratio of the mean OD of *HIF-1α* over *β-actin* was expressed as comparative level of *HIF-1α* mRNA.

In situ hybridization

The rats were anesthetized and subsequently perfused trans-cardiac with 4% freshly prepared phosphate-buffered paraformaldehyde (PFA). The spinal cord was removed and fixed in 4% PFA for 2–4 h. After rinsing in PBS twice and one night of cryoprotective treatment in 20% sucrose, horizontal sections of 6 μm were cut in a cryostat and mounted on slides coated with 3-aminopropyltriethoxysilane, then dried at 37°C for 1–2 h prior to ISH. The sections were dehydrated in a series of ethanol gradients of 30, 60, 80 and 90% before being kept at −80°C.

HIF-1α RT-PCR product described above was sequenced and inserted into pGEM-T easy vector (Promega). Probes were prepared by run-off. Briefly, the recombinant plasmid was digested to allow transcription from T_7 promoter for the anti-sense probe and SP6 for the sense probe. At the same time, the probes were labeled with digoxigenin-11-dUTP during *in vitro* transcription according to the DIG RNA Labeling Kit (Borehringer Mannheim). Labeled probes were purified by ethanol precipitation and their concentration was determined by quantification against known standards on Hybond H^+ filters, as described in the DIG system user's guide for filter hybridization (Borehringer Mannheim).

The tissue sections were rehydrated in gradient ethanol before being rinsed in PBS twice at room temperature and digested with Protease K at 37°C for

15 min. Then the tissue sections were refixed with 4% PFA for 10 min, followed by washing twice in PBS and once in 2 × SSC. The sections were dehydrated in ethanol series and air dried. After prehybridization at 55°C for 1 h in prehybridization solution, exact amount labeled RNA probes were added and the sections were hybridized at 55°C for 18–24 h. After that, the sections were washed with wash buffer for 30 min at 55°C, digested with 20 μg/ml RNase for 30 min at 37°C to remove the unbound RNA probe and then incubated overnight at 4°C with a 1:1000 dilution of anti-DIG alkaline phosphatase antibody and 1% normal goat serum. The sections were washed again with alkaline phosphatase buffer twice and developed with the appropriate amount of NBT/BCIP. After the reaction of staining finished, the entire cell nuclei would be restained with methyl green. The nuclei in positive cells were stained with purple.

Immunohistochemistry
The same fixed spinal cord samples with the method described in ISH were embedded with paraffin. Then they were cut into 5 μm sections and kept for future use. Since IHC was performed, these sections were deparaffined with dimethylbenzene, rehydrated in ethanol series and then treated with 3% H_2O_2 to inactivate the internal catalase prior to antigen retrieval. Thereafter, the sections were incubated overnight in 1% BSA, 1% normal goat serum, 0.3% Triton X-100 and 1:100 monoclonal antibody against HIF-1α and VEGF (NeoMarkers Biologic) in PBS at 4°C. After rinsing three times with 0.3% Triton X-100 in PBS, the sections were shaken in 1:1000 biotinylated anti-rabbit IgG (Sigma). Then the sections were overlaid with steptavidin-biotin-HRP (horseradish peroxidase, Sigma) for 2 h, which was followed by rinsing in 0.3% Triton X-100 in PBS. The sections were developed in 50 mM Tris-HCL (pH 7.4) for 5 min. Substrate of HRP (DAB and H_2O_2) was added for the staining reaction according to the manual of Sigma and the cytoplasm with yellow staining would be identified as positive cells. Additionally, the sections from different time intervals of postinjury were also stained with hematoxylin–eosin (HE).

Enzymehistochemistry
Enzymehistochemistries to ALDA and LDH-M were performed according to the Neupreux and Wegmann Method and the Jacobsen Method, respectively, which were described by Zhu.[17] Each case was performed with three parallel sections and the mean OD of random 100 positive cells in each section was analyzed with BioMias2000.

Statistical analysis
Each section was plotted into nine panes and the numbers of positive cells in ISH and IHC were counted in invariable three among them. All data were expressed as mean ± SEM. The results of ISH, IHC and EHC were analyzed using SPSS10.0. Significant differences among experimental time points were analyzed within a level using a one-way ANOVA. Differences were considered to be statistically significant at the level of $P < 0.05$.

Results

Expression pattern of HIF-1α in SCI
The goal of this study was to test the hypothesis that HIF-1α is involved in the development of SCI. RT-PCR was performed with RNAs from different times (0, 6, 12 h, 1 day, 2 days, 3 days and 1 week) after SCI as templates. After a 25-cycle reaction, the PCR products of 443 bp for *HIF-1α* and 956 bp for *β-actin* in each reaction mixture could be observed by gel electrophoresis (Figure 1). Taking the ratio of the mean OD of *HIF-1α* over internal control *β-actin* as the comparative level of *HIF-1α* mRNA (in Table 1), the trend of *HIF-1α* mRNA could be easily shown: *HIF-1α* was retained at a low level in normal spinal cord; in 6–72 h after SCI, the expression of *HIF-1α* significantly increased; after 1 week, its expression began to decrease even though the amount was still above normal. It was also noticed that the change of *HIF-1α* expression was concurrent with the transformation of ischemic situation after SCI: especially after 2–3 days, the most severe clinical ischemic symptoms appeared, at the same time as the expression of *HIF-1α* reached its maximum as assayed by RT-PCR.

To further determine the distribution pattern of HIF-1α in neural cells in the development of SCI, we performed ISH and IHC on tissue sections from different times. At least three sections were prepared for each sample so that HE staining was completed to confirm pathologic change (Figure 2): the extensive bleeding spots in the injured spinal cord appeared within the initial 24 h. From 24 to 72 h, edema became obvious and a large number of neutrophiles soaked in involved tissues. At the same time cystic degeneration could be easily observed in the dorsal column of the injured

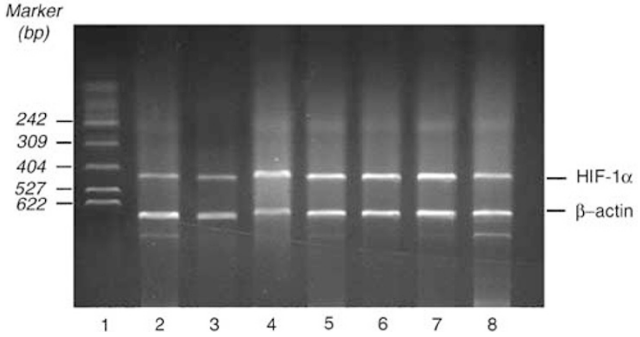

Figure 1 The electrophoresis of *HIF-1α* RT-PCR products. Lanes 1–8 represent the DNA Marker, normal group, 6, 12 h, 1 day, 2 days, 3 days, 1 week after SCI, respectively. *β-actin* is taken as control

spinal cord. All the pathologic phenomena suggested that it could be the most distinct phase of ischemia and hypoxia. From 72 h to 1 week, the pathologic section showed that the course of disease developed the chronic phase and there were a number of large macrophages and small vessels expanded followed by the hyperplasia of endothelia cells of vessels and increased density of capillary vessels.

As for the change of HIF-1α during SCI, ISH results are shown in Figure 3. Almost all the positive signals of *HIF-1α* mRNA with antisense probe were detected in neural cells on the sections from postinjury, with only weak or even no positive signal in normal spinal cord. Besides the neural cells, ependymal cells and endothelial cells from capillary vessels also expressed *HIF-1α* mRNA remarkably. Furthermore, among all the sections from postinjury, *HIF-1α* mRNA was most significant after 3 days, which is consistent with the result from RT-PCR. The moderate expression lasted 1 week. No detectable signals could be obtained with the sense probe as the negative control (data not shown). On the protein level, IHC showed that the trend of HIF-1α expression fitted with the result of ISH but the increase of HIF-1α protein was earlier than its mRNA: protein of HIF-1α increased apparently in 6 h after injury, and it reached its maximum within 24 to 48 h (Figure 4). Statistical analysis of the quantitative measurements in ISH and ICH showed that the expression of HIF-1α significantly changed during the observed postinjury phase ($P<0.05$).

Table 1 The RT-PCR results of *HIF-1α*

Groups	*OD values (HIF-1α)* $\bar{x}\pm SD$	*OD odds (HIF-1α/β-actin)*
Normal	119.74±2.3	0.68
6 h after SCI	107.19±3.91	0.78
12 h after SCI	176.36±4.21	0.93
1 day after SCI	188.62±6.34	1.07
2 days after SCI	190.80±4.52	1.23
3 days after SCI	201.69±7.85	1.17
1 week after SCI	156.20±3.94	0.93

Analysis of glycolytic enzymes activity in injured spinal cord

At least four kinds of glycolytic enzymes have been reported as the target genes of transcriptional factor HIF-1α,[18] and the involvement of HIF-1α that has been shown in the experiments described above further raised our interest in the activity changes of the most important glycolytic enzymes, ALDA and LDH-M, in the SCI model.

The results of EHC are shown in Figure 5. In the normal spinal cord, the activities of ALDA or LDH-M were relatively low, which were enough to meet the request of normal energy metabolism. However, their activities increased during the first week in the SCI

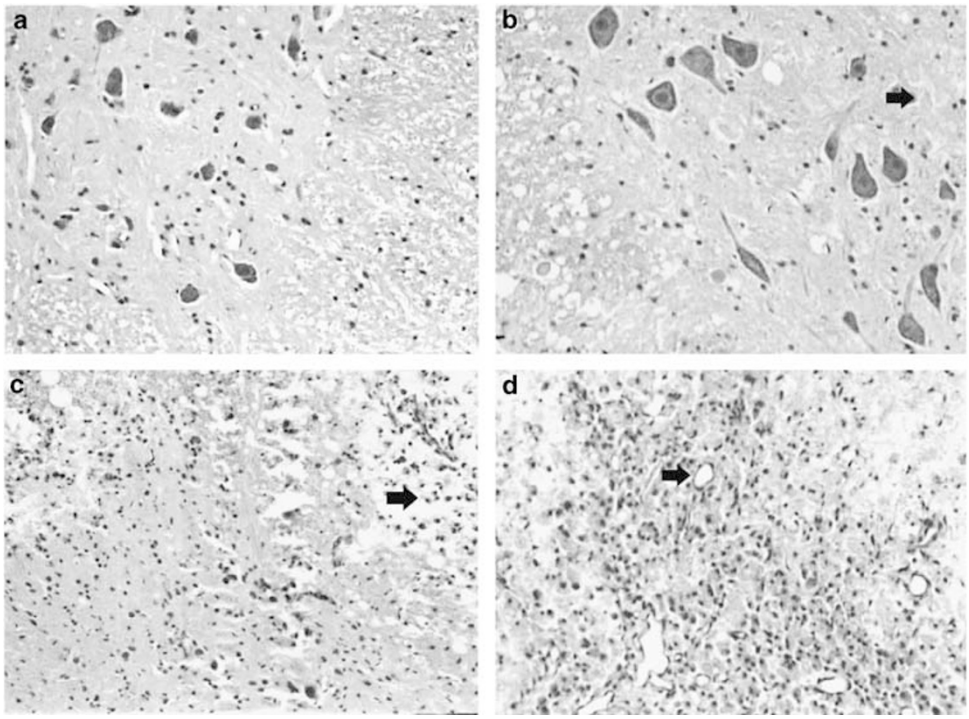

Figure 2 The hematoxylin–eosin (HE) staining. (**a**) The normal spinal cord. (**b**) The injured spinal cord in 12 h, arrow points to the bleeding spots. (**c**) The injured spinal cord in 72 h, arrow points to cystic degeneration. (**d**) The injured spinal cord in 1 week, arrow points to angiogenesis ×200

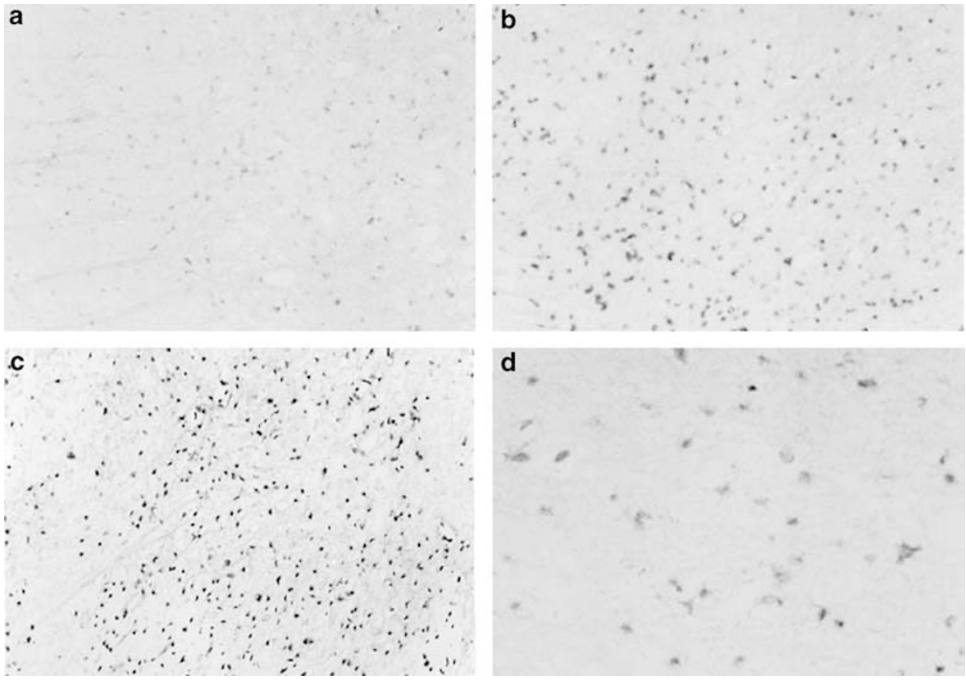

Figure 3 The ISH of *HIF-1α* mRNA. The positive cells were stained with HRP, while all the cell nuclei were re-stained with methyl green. (**a**) Expression of *HIF-1α* in normal and almost no obvious signals were detected. (**b**) The increased expression of *HIF-1α* in 6 h after SCI. (**c**) The prominent expression of *HIF-1α mRNA* on day 3. (**d**) The moderate increased expression of *HIF-1α* retained till 1 week ×200

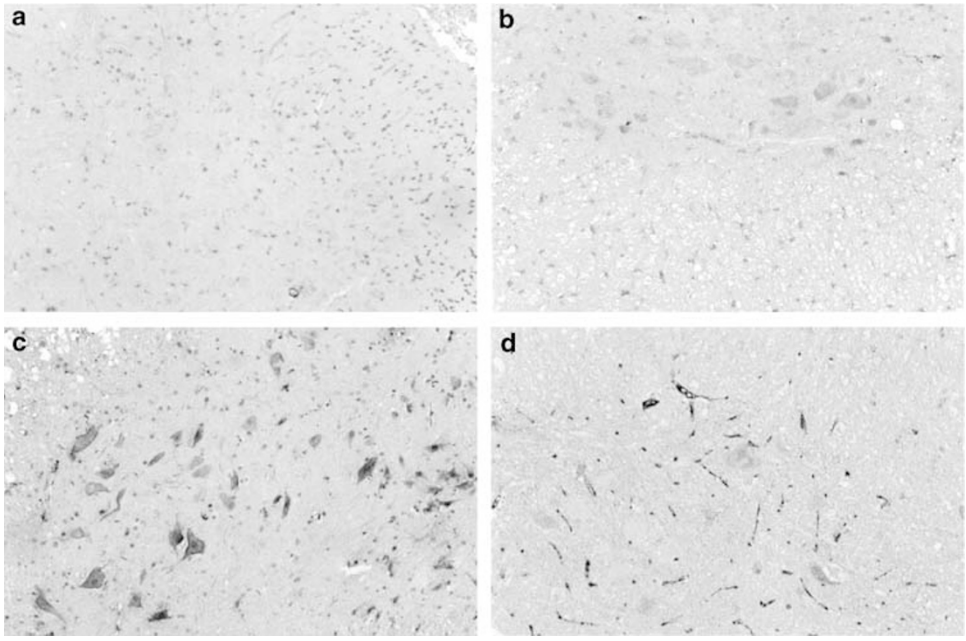

Figure 4 The IHC of transcriptional factor HIF-1α. The cytoplasm of positive cells was stained yellow with DAB and all cell nuclei were re-stained with hematoxylin. (**a**) Expression of protein HIF-1α in normal and no obvious signals were observed. (**b**) The increased expression of HIF-1α at 6 h after SCI. (**c**) The expressional peak of protein HIF-1α was brought forward to 48 h after SCI. (**d**) The expression of HIF-1α after 1 week of SCI ×200

model ($P<0.05$). Especially after 3 days the activities of both ALDA and LDH-M seemed to reach their maximum, which was also consistent with the severe symptoms of ischemia. However, the activity peaks of ALDA and LDH-M were delayed when compared with their regulatory protein of HIF-1α.

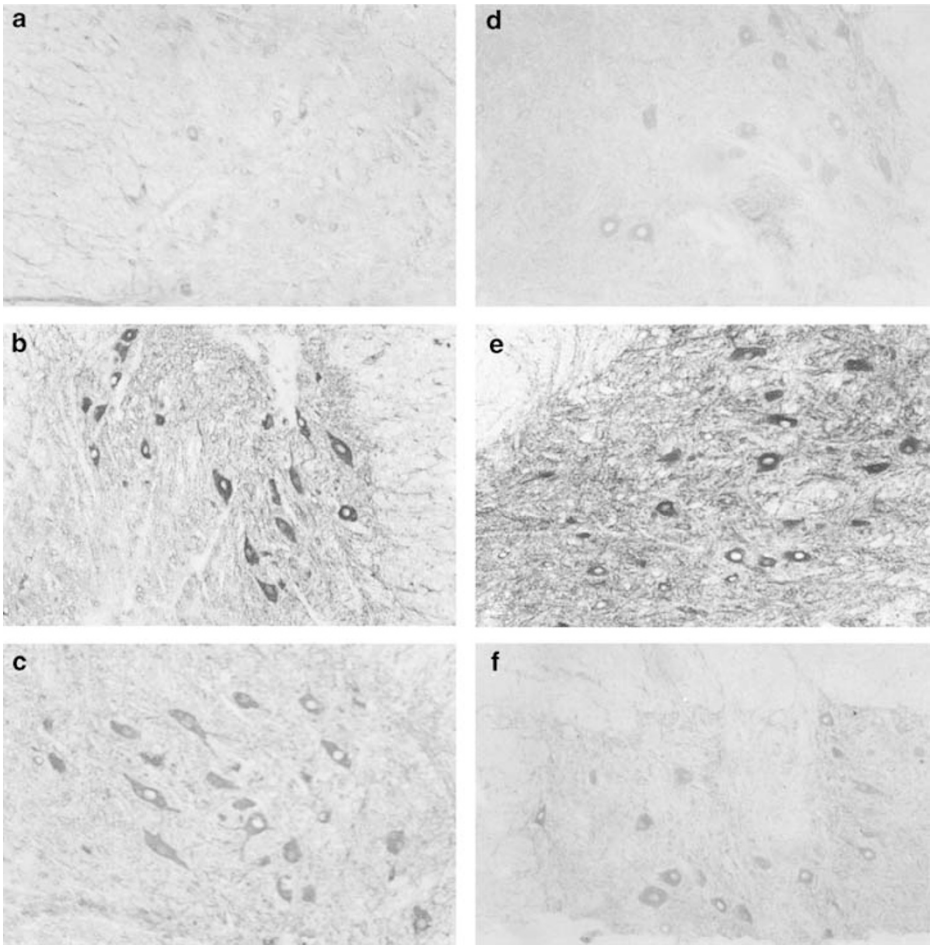

Figure 5 The EHC of ALDA and LDH-M. Among them, (**a–c**) represented the detected activities of ALDA in normal, on 3 days and 1 week after SCI, while (**d–f**) represented the enzymes activities of LDH-M in normal, on 3 days and 1 week after SCI $\times 100$

Microcirculation re-establishment and the expression of associated gene of VEGF in SCI

Re-establishment of microcirculation plays an important role to ameliorate ischemia and hypoxia. In our study, the shaping of blood vessels was observed in the involved tissues after 3 days and it was revealed that less or even no obvious liquefied cyst was formed in the regions of microcirculation re-establishment. Thus, *VEGF*, which is the best candidate for angiogenesis and also another target gene of HIF-1α, was studied to find out if it participated in microcirculation re-establishment in the SCI model.

Immunohistochemisty confirmed the involvement of VEGF and the change of the expression pattern in SCI (Figure 6). VEGF was expressed at a low level in the normal spinal cord; in the rodent model of SCI, its expression increased obviously after 24 h; within 48–72 h, it reached its peak and began to decrease rapidly. Additionally, the cell types involved in SCI were extended to most neurons, glia cells and phagocytes except for the epithelia of ependyma, astrocytes and some specific neurons in normal. Compared with its regulatory protein of HIF-1α, the delayed peak of VEGF was also observed. Similarly, analysis using a one-way ANOVA revealed that the difference of VEGF expression was statistically significant.

Discussion

Under oxygen tension, it has been demonstrated that a variety of cells and tissues could express HIF-1α significantly in contrast with the low level of HIF-1α protein for the short half-life of less than 5 min in normal.[19] But the fact that CNS could express HIF-1α was first revealed when it was detected in the ischemic brain resulting from permanent middle cerebral artery occlusion.[11] Since ischemia and the subsequent hypoxia are the focus of secondary SCI, here we hypothesized that *HIF-1α* and its target genes should be involved in the biological process of the injured spinal cord and that their involvement could have an important impact on the courses of the disease.

In our study, a compressive model causing SCI was adopted to investigate the relationship between the pathophysiological process and the expression pattern of involved genes. Through the gross and microscopic observation, we found that the pathological procedures in our model were the same as those used in the previous

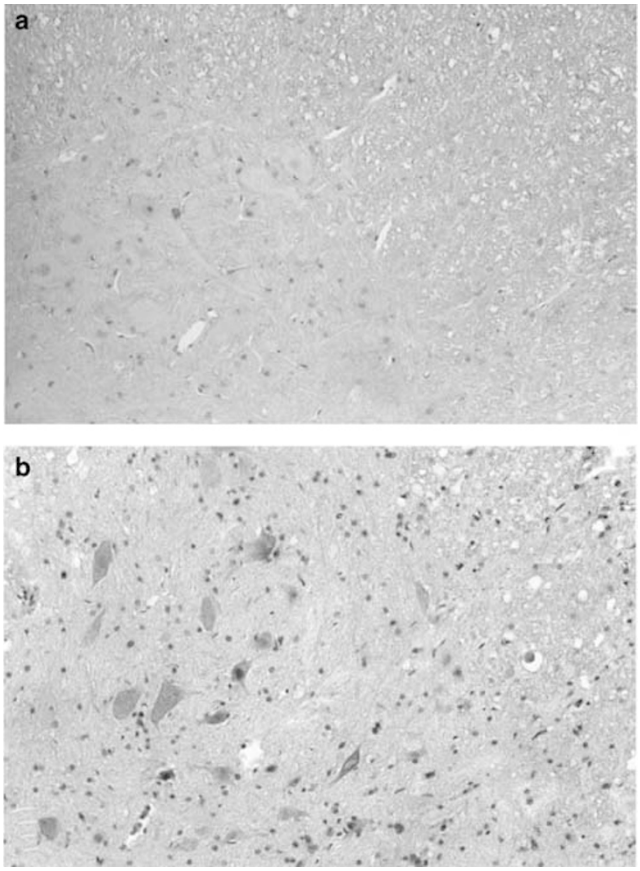

Figure 6 The IHC results of VEGF. The cytoplasm of positive cells was stained yellow with DAB and cell nucle re-stained with hematoxylin. (**a**) No detected signals of VEGF in normal spinal cord. (**b**) The increased expression pattern of VEGF on day 3 after SCI $\times 200$

studies: from the instant to 3 days of SCI, progressive insults including the appearance of ischemia could be seen in the injured and adjacent segments, accompanied by the increasing severity of neurological dysfunction. These results have shown that the lack of collateral circulation,[20] the injury of microvessels, the spasm of microarteries[21] and the formation of microthrombi[22] could distinctly decrease blood flow in the injured spinal cord and subsequently result in severe ischemia. After 3 days, a serial procedure of repair, which was testified to be associated with the increase of HIF-1α and its target genes, began to emerge in the involved region.

Compared with the previous studies showing that HIF-1α protein significantly increased in 1–7 days after SCI,[15] our study uses more accurate time intervals and analyzes the expression changes at the mRNA level. The accumulation of HIF-1α protein in hypoxic environment may be caused by two means: (1) increasing stability of protein, namely the prolonged half-time of HIF-1α, (2) activating *HIF-1α* transcription to increase the synthesis of protein. But these two regulatory mechanisms do not always exist in the same physiologic process. In this study, the potential regulatory mechanisms of HIF-1α were suggested by comparing the expression pattern between mRNA and protein. By using RT-PCR and ISH, we showed the significant increase of *HIF-1α* transcription: the transcription of *HIF-1α* began to be activated in 6 h after injury, reached its climax on 3 days and then declined slowly, which confirmed to us that the regulatory means of activating transcription was employed in the SCI model. We synchronously detected the protein of HIF-1α with monoclonal antibody, and interestingly the expression peak of HIF-1α protein (24–48 h after injury) was earlier than that of mRNA even though the increased protein expression partially overlapped with that of the mRNA. This peak of protein HIF-1α cannot be explained only by the increase of transcription and is partially owed to the other regulatory mechanism of the prolonged half-life of protein HIF-1α. So the emergence of HIF-1α, as an index of hypoxia, indicated that the injured spinal cord underwent oxygen stress and that it upregulated a series of target genes whose functions could compensate for the detrimental effects of ischemia.

The enhancement of glycolysis and re-establishment of microcirculation are all important pathways to relieve oxygen stress. Glycolysis can supply most of the energy needed by the cells in CNS during hypoxia, and the modulation of microcirculation and neovascularization is sometimes the terminal way to solve the local hypoxia caused by insufficient perfusion. Although some of the genes regulated by HIF-1α, which are involved in these two compensatory physiologic processes, have been previously reported, the role HIF-1 plays in the transcriptional regulation of gene expression in response to hypoxia may be both cell type and gene specific.[7] We set out to identify the expressional patterns of glycolytic enzymes including ALDA and LDHM as well as the angiogenic molecule VEGF in SCI, as the reported target genes of HIF-1α, in SCI.

In fact, glycolysis can serve as a partial supplier of energy in normal CNS, which seemed to be proved by our results that the enzymes of glycolysis retained low detectable levels in normal controls. To the cells suffering hypoxic environment in SCI, the modulation of glycolysis should be an important adaptive response to help cells retain energetic metabolism and fundamental physiologic function by increasing the amount of the glycolytic enzymes and enhancing the activity of glycolysis. It is known that glycolysis is a series of reactions in which 11 glycolytic enzymes participate.[23] As a transcription factor involved in the response to hypoxia, HIF-1α has been identified as an activator of the transcription of at least four glycolytic enzymes including ALDA, PGK1, PKM and LDHM.[18] So, we investigated the enzyme activities of ALDA and LDHM with EHC and the possibility that HIF-1α activates transcription of glycolytic enzymes in SCI. Our experimental results indicated significant increases enzyme activities of these two glycolytic enzymes existing in all types of cells in the injured spinal cord. But the modulations of these enzyme activities are achieved in two ways: allosteric effectors and enzyme synthesis. According to our data, the upregulation of the two

glycolytic enzyme activities lasted for days, which could only be explained by an increase of enzyme synthesis. At the same time, it is worth noticing that the upregulation of the two glycolytic enzymes activities took place after that of HIF-1α protein, which suggested that HIF-1α participates in the transcriptional activation of glucolytic enzymes in SCI combined with the fact that the sequences from the regulated glycolytic enzymes contain the special sequences – hypoxia response element (HRE) that the HIF-1α protein binds to.[18]

Neovascularization is an important way for ischemic tissue to regain blood flow. In Zhang's study, the secondary lesion of the spinal cord declines along with the revascularization of the involved tissue.[24] This evidence suggested that revascularization precedes spinal tissue repair and nerve regeneration, and may ameliorate the cascade of progressive tissue necrosis. This process is believed to be associated with some angiogenic molecules, among which VEGF is the most important one that is mitogenic for endothelial cells. In normal CNS, VEGF is mainly expressed in the epithelial cells of the choroids plexus, astrocytes and some neurons.[25] In the model of brain ischemia, the upregulation of VEGF was detected in the peumbra, which proved that VEGF was involved in the neovascularization of CNS.[13] In our study, the revascularization can be visible from 3 days after injury when iNOS (data not shown) and VEGF were both detected and the expression of these two factors extended in most neurons, glial cells and some inflammatory cells. Moreover, dynamic change of the expression pattern of VEGF after SCI is similar to that of glycolytic enzymes. We speculated that HIF-1α could act as a critical regulatory factor for its target genes associated with the modulation of glycolysis and re-establishment of microcirculation in SCI.

Conclusion

Our present study showed the that injured spinal cord that underwent insulting ischemia after SCI responded with the increase of transcriptional factor HIF-1α and its target genes such as glycolytic enzymes and angiogenesis molecular VEGF, and the dynamic expression changes of these genes in hypoxia are assumed to help neural cells adapt to a detrimental situation.

Acknowledgements

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