pEGFR promotes the neural function recovery after decompression of compressed spinal cord injury

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Abstract

Purpose Investigating the roles of phosphorylated epidermal growth factor receptor (pEGFR) in the recovery of neural function after decompression of CSCI, therefore provide experimental basis for the development of therapeutic strategies and medicines for treating CSCI.

Methods A CSCI model was established with a customized device, and was then subjected to spinal decompression. The motor functions were monitored by the Basso, Beattie & Bresnahan (BBB) locomotor rating scale; the number of axonal myelinated fibers was estimated by staining with luxol fast blue (LFB); pEGFR and phosphorylated Akt1 (pAkt1) were detected by Western blot; pEGFR+NG2+(NG2+ cells are precursor to oligodendrocytes and pAkt1+NG2+ cells were detected by double-labeling immuneuorescence assay.

Results After decompression of CSCI, the BBB scores and the number of myelinated nerve fibers gradually increased with time. Meanwhile, the expression of pEGFR and pAkt1 were up-regulated and the number of pEGFR+NG2+ and pAkt1+NG2+ cells increased consistent with the changes of motor functions and the number of myelinated nerve fibers. Whereas, significant decreases in BBB scores, expression level of pAkt1, as well as numbers of myelinated nerve fibers, and pAkt1+NG2+ cells were observed after inhibition of expression.

Conclusions Up-regulated expression of pEGFR can promote recovery of neurological functions in rats with CSCI. This effect is achieved by activation of pAkt1 a downstream signal molecule of pEGFR, which subsequently promotes the proliferation of oligodendrocyte precursor cells (OPCs).

Background

CSCI is usually caused by spinal tumors, spinal tuberculosis, vertebral fractures, epidural hematoma or other diseases [1–4]. Previous studies showed that demyelinating lesion is one of the important pathological changes of CSCI, while the apoptosis of OPCs is the main reason for demyelination after CSCI [5–10]. The nerve impulses of demyelinating nerve fibers become diffused during conduction, preventing the electrical conduction of axons and resulting in partial or complete inhibition of movement, sensation and sphincter functions below the level of injury [11–14]. Basic research and clinical observations showed that a certain degree of recovery in neurological functions are possible after decompression of CSCI [11, 12, 15, 16]. However, the detailed mechanisms of neurological recovery after decompression of CSCI have not been fully elucidated. Therefore understanding of these specific mechanisms is of great significance for developing new medicines and treatment strategies. Epidermal growth factor receptor (EGFR, ErbB–1 or HER1) is one of a member of the epidermal growth factor receptor (HER) family [17]. Previous studies showed that pEGFR can promote the proliferation and differentiation of OPCs, thereby protecting the integrity of myelin sheath [18–20] and suggesting that the activation of EGFR may promote the recovery of neurological functions. Therefore, we hypothesize that
pEGFR fosters recovery of neural functions by promoting the proliferation and differentiation of OPCs after decompression of CSCI.

In this work, we revealed the relationship between the expression level of pEGFR and the number of myelinated nerve fibers, the BBB scores, the expression level of pAkt1 and the number of pAkt1⁺-NG2⁺ cells after decompressed of CSCI in rats. Taken together, we probed the mechanisms of neural function recovery and provided experimental basis for the development and formulation of novel therapeutics after decompression of CSCI.

Materials And Methods

Study design

A total of 140 Sprague-Dawley (SD) rats weighing 250 g to 320 g were randomly divided into three groups: (1) normal group (Nn = 20); (2) model group:, which was divided into the following subgroups: model 0 day group (D0n = 20), model 1 day group (D1n = 20), model 7 day group (D7n = 20), model 14 day group (D14n = 20), model 21 day group (D21n = 20); (3) intervention group (In = 20). All rats were provided by the Experimental Animal Center of Chongqing Medical University. All experimental procedures were performed in accordance with the Animal Care and Ethics Committee and were approved by the Ministry of Science and Technology of the People's Republic of China. The rats were housed in a 26°C room on a 12:12 dark/light cycle and were supplied with sufficient food and water.

Surgical procedure

Model groups: the CSCI model (Additional File 1) was constructed based on a previous method developed by our group [5, 6, 21]. The rats were fasted 24 hours prior to surgery, anesthetized by intraperitoneal injection of 3.5% chloral hydrate and subjected to continuous oxygen inhalation. The rats were then fixed prone on a surgical table. The anterior and the posterior articular processes were excised without damaging the spinal cord. A small rectangular stainless steel board (3mm×2mm) was placed in the center of the spinal cord surface at L1. A custom-made swallow tail-like stainless steel fixation device was positioned between T12 and L2. To establish a rat model with a compressed spinal cord, the center of the stainless steel fixation device was inserted using a small screw (2 mm in diameter and 2 mm long with a flat and smooth screw tip) slowly and vertically until the front end of the screw reached the small rectangular stainless steel board. The incision was then gradually sutured in layers. The rats were allowed to recover from anesthesia and housed as described previously. Thereafter, the screws were turned inside 1/4 circle every 4 days until occurrence of double lower limbs paralysis and incontinence. After successful model construction, spinal cord decompression was performed. At 0, 1, 7, 14 and 21 days after decompression, the rats from different groups were anesthetized. Physiological saline (250 mL, 4°C) and 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS 500 mL, 4°C, pH 7.4)
were perfused into the left ventricle and outflowed from right auricle. When the right auricle efflux became clear, spinal cord sections from L1 (1cm) were quickly harvested.

**Normal group:** the rats were not subjected to any experimental procedure.

**Intervention group:** after successful model construction and decompression, pEGFR inhibitor (0.6mg/kg•d, ab141839/PD153035) was injected intraperitoneally and the spinal cord was removed at day 14.

**Neurological function assessment**

Locomotor activities were examined based on the BBB rating scale in an open field, according to the indications of published articles by two independent observers in a double-blind manner[22, 23]. A total of 21 points from 0 (complete paralysis) to 21 (normal) were recorded after decompression of CSCI.

**Luxol fast blue staining**

The spinal cord sections were removed and fixed in 4% paraformaldehyde for 24 hours at 4°C. Sections from the center of the injury were selected along with those from 0.5cm to 0.7cm rostral and caudal to the injury site. The tissues were then dehydrated successively in 10%, 20% and 30% sucrose solutions, and embedded in optimal cutting temperature (OCT) compound. Transverse sections (10 µm in thickness) of the spinal cord were prepared on a frozen slicer. Spinal cord sections were stained with luxol fast blue at 60°C for 2h. The sections were then rinsed with 95% ethanol and differentiated in 0.05% lithium carbonate solution followed by 70% ethanol. Differentiation was stopped by distilled water until unmyelinated tissue turned white. Twelve random micrographs from the lateral funiculus were obtained under an Olympus microscope with an objective lens of 40×. Images of at least 10 randomly sections were captured. The total number of myelinated fibers in the micrographs was further determined using the Image-J software (National Institutes of Health, USA) by a blinded investigator.

**Double-labeling immunofluorescence assay**

To detect co-expression of p-EGFR, pAkt1 and NG2+ (the marker of OPCs) in the spinal cord sections, we used the primary antibodies listed in Table 1 (see supplemental materials). Tissue sections were rewarmed, rinsed and incubated in 5% donkey serum (Jackson ImmunoResearch, Lancaster, PA, USA) for...
1 h at 37°C in a humidified atmosphere to permeabilise the tissue and block non-specific protein-protein interactions. The tissues were then incubated with the primary antibody overnight at +4°C. The tissue sections were rinsed again with 0.01mol/L PBS. The secondary antibody (red) was cy3-conjugated goat anti-rabbit IgG (H+L) used at a 1/200 dilution for 1.5h at 37°C in a humidified atmosphere in the dark. Alexa Fluor 488 goat anti-mouse IgG (H+L) was used to label NG2 (green) at a 1/200 dilution for 1.5h at 37°C in a humidified atmosphere in the dark. A nuclear dye (4′,6-diamidino–2-phenylindole, 1:20; Bestbio Inc., China) was used to stain the cell nuclei (blue) for 5 min. The tissue sections were then washed and mounted in 50% glycerol dissolved in PBS. The samples were observed under a confocal microscope (Leica TCS SP2, Germany). All the digital images from lateral funiculus were captured in a double-blind manner from four random fields per section of the injured epicenter of the cross sections in the rats. The number of p-EGFR+-NG2 and pAkt1+-NG2 signals per field were counted for further analysis.

**Western blot**

Tissues soaked at 4°C in a buffer containing 50 mmol/L ethylenediaminetetraacetic acid, 2μg/mL leupeptin, 2μg/mL pepstatin A, 2 mmol/L phenylmethanesulfonyl fluoride and 200 KIE/mL aprotinin were broken down mechanically using a blender. The homogenates were then centrifuged at 10,000×g for 20 min at 4°C. The supernatant was collected, and protein concentration was determined using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The proteins of the sample were separated using 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The blotted membranes were incubated in 5% skim milk to block non-specific protein-protein interactions. For immunoblotting, the following primary antibodies were used: polyclonal rabbit anti-AKT1 antibody (1:1000; Abcam, Cambridge, UK, ab66138) and monoclonal rabbit anti-EGFR antibody (1:1000; Abcam, Cambridge, UK, ab52894). Alkaline phosphatase-conjugated anti-IgG antibody (1:10000, Santa Cruz Biotechnology) was used as the secondary antibody. Immunoreactive bands were visualized using a chemiluminescent substrate (Pierce Inc., Rockford, IL, USA). Western blot bands were quantified by a gel densitometry (Bio-Rad). The ratio of protein-to β-actin was obtained for each sample, and each point was measured in triplicate.

**Statistical Analysis**

Statistical analyses were performed using the SPSS Statistics 20.0 software (IBM, Inc., USA). Number of Myelinated nerve fibers, and expression level of pEGFR and pAkt1 proteins for the specimens were expressed as means±SD. Differences between individual groups were initially compared using one-way ANOVA. The data were then analyzed with LSD multiple-comparison post hoc test. Differences between the intervention group and model group on day 14 were compared using independent samples t test. All of the reported P values were two-sided, and P < 0.05 was considered statistically significant.

**Results**
Neurological function assessment

The rats in the CSCI group were found to become paralyzed and incontinent. The mean BBB scores in the CSCI group were lower than those in the normal group (model group vs. normal group, P = 0.000 or P < 0.05, Figure 1 and Additional File 2). These results validated the custom-designed model of CSCI as a suitable model system for functional studies of clinical CSCI. The BBB scores of the rats decreased remarkably and reached the minimum value on day 0 or day 1 (D0 group vs. D1 group, P>0.05; D0 group vs. other model groups (D7, D14, D21 group), P = 0.000 or P < 0.05; D1 group vs. other model groups (D7, D14, D21 group), P = 0.000 or P < 0.05, Figure 1 and Additional File 2). Thereafter, the BBB scores of the rats were found to gradually increase with increasing irradiation time (D0 group vs. D7 group, P = 0.000 or P < 0.05, D0 group vs. D14 group, P = 0.000 or P < 0.05, D0 group vs. D21 group, P = 0.000 or P < 0.05, D1 group vs. D7 group, P = 0.000 or P < 0.05, D1 group vs. D14 group, P = 0.000 or P < 0.05, D1 group vs. D21 group, P = 0.000 or P < 0.05; D7 group vs.D14 group, P = 0.000 or P < 0.05; D7 group vs.D21 group, P = 0.000 or P < 0.05, Figure 1 and Additional File 2).

Number of myelinated nerve fibers

Myelinated nerve fibers were visualized using luxol fast blue (LFB) and the number of myelinated nerve fibers was counted by Image J (National Institutes of Health, USA). Normal distribution data were analyzed by one-way ANOVA and the differences between control groups were tested by least significant difference (LSD). The results indicated that after decompression of CSCI, the number of myelinated nerve fibers in the model group was significantly lower than that in the normal group (model group vs. normal group, P = 0.000 or P < 0.05, Fig.2 and Additional File 3), while the number of myelinated nerve fibers in the model group gradually increased with time (D7 group vs. D0 group; D7 group vs.D1 group; D14 group vs.D0 group; D14 group vs.D1 group; D21 group vs.D0 group; D21 group vs.D1 group, P = 0.000 or P < 0.05, Fig.2 and Additional File 3). Independent samples t-test revealed that the number of myelinated nerve fibers in the intervention group was significantly lower than that in the 14 day group (D14 group vs. I group, P = 0.000 or P < 0.05, Fig.2 and Additional File 2).

Double-labeling immunofluorescence assay

The pEGFR-NG2+ and pAkt1-NG2+ cells were scattered in the white matter in the N, D0 and I samples, while the density of pEGFR-NG2+ and pEGFR-NG2+ cells increased significantly in the D14 group. One-way ANOVA analysis was performed followed by LSD post hoc tests where appropriate (D14 group vs. N group, D0 group or I group, P = 0 or P<0.05, Fig.4 and Fig.6).

Western blot
One-way ANOVA analysis was performed followed by LSD post hoc tests where appropriate, The expression levels of pEGFR and pAkt1 were low in the N, D0 and D1 groups. Compared with the N, D0 and D1 groups, the expression of pEGFR and pAkt1 was significantly up-regulated in the D7, D14 groups and reached the peak at D14 ($P < 0.05$, D7 group vs. N group, D7 group vs. D0 group, D7 group vs. D1 group, D14 group vs. N group, D14 group vs. D0 group, D14 group vs. D1 group; $\&P < 0.05$, D7 group vs. D1 group, D14 group vs. D7 group). Results of independent samples t-test indicated that the expression levels of pEGFR and pAkt1 in the intervention group were significantly lower than that in the 14 day group (*$P< 0.05$, D14 group vs. I group, Fig.3, Fig.4 and Additional File 4–7).

**Discussion**

Our findings suggested that pEGFR promotes the recovery of neural functions after decompression of CSCI. This effect is achieved by activation of pAkt1, and the subsequently stimulation of OPCs proliferation and differentiation.

Compressed spinal cord injury (CSCI), typically caused by vertebral fractures, spinal tumors, spinal tuberculosis or other spinal canal occupying lesions, has become a global issue\[3, 10, 13, 24–26\]. CSCI can cause severe neurological dysfunction, pose enormous physical pains and psychological stresses for patients, and exert bring heavy financial burdens to families and society \[13, 26, 27\]. Previous studies have indicated that a certain degree of nerve function recovery can be achieved after spinal cord decompression and spinal stabilization, but the recovery outcomes are still far from meeting the needs of daily activities\[28, 29\]. Therefore, it is of great theoretical and potential practical values to elucidate the mechanism of neural function recovery after decompression of CSCI.

Myelinated nerve fibers are formed from oligodendrocytes (OLs), which support axons and provide electrical insulation in the form of myelin sheath wrapped around axons\[30, 31\]. Oligodendrocyte precursor cells (OPCs) are the main source of OLs. Previous studies have showed that the loss or lack of OPCs, and consequent lack of differentiated oligodendrocytes, are associated with loss of myelination and subsequent impairment of neurological functions\[32–35\]. Previous research indicated that the demyelination caused by degeneration and necrosis of spinal cord OLs is the key link of CSCI dysfunction; contrarily, the increase of myelinated nerve fibers is beneficial to the recovery of neurological functions after CSCI\[5, 6, 10\]. Our findings are in accordance with previous studies, which suggested that the recovery of nerve functions recovery after decompression of CSCI is closely related to the increase of the number of myelinated nerve fibers. However, the detailed mechanism for the increase of myelinated nerve fibers after decompression of CSCI has not been elucidated. Therefore, the in-depth exploration of this mechanism is of substantial theoretical and practical significance.

In this study, we investigated the protein factor that promotes the proliferation of OPCs. Epidermal growth factor receptor (EGFR) is a transmembrane protein that is activated by binding of its specific ligands. Upon activation of EGFR by its growth factor ligands, autophosphorylation of several tyrosine (Y) residues in the C-terminal domain of EGFR occurs \[16, 17, 22\]. The autophosphorylation elicits
downstream activation and signaling by several other proteins that interact with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains. These downstream signaling proteins initiate a number of signal transduction cascades, principally the Akt, MAPK, and JNK pathways, leading to DNA synthesis and cell proliferation[36–39]. The role of pEGFR in promoting cell proliferation and differentiation has been confirmed in many diseases, such as schizophrenia, depression disease, hyperplasia and tumorigenesis[19]. The results of Figs. 1–4 jointly indicate that up-regulation of pEGFR expression can promote the proliferation and differentiation of OPCs, and thus benefits the recovery of neural function in rats with CSCI. However, this conclusion is incompatible with those of several previous studies. Li et al. reported that inhibition of EGFR can ameliorate excessive astrogliosis, as well as improve the regeneration microenvironment and functional recovery in adult rats [40]. Erschbamer et al. also reported that inhibition of EGFR following spinal cord injury improves structural, locomotor, sensory, and bladder recovery from experimental spinal cord injury[41]. These observations may be attributed to the following reasons. First, the level of spinal cord injury is different. It is well recognized that the responses of various parts of the central nervous system to injuries can be completely different. The injured site of the models in Li et al. and Erschbamer et al. was located in the thoracic segment, while the injured site in our experiment was in the lumbar segment. Second, different inhibitors were used in the experiments. Because of the different lots of EGFR inhibitors, Kelli Sharp et al. failed to confirm the report of Erschbamer et al. that treatment with an EGFR inhibitor after spinal cord injury increases tissue sparing and improves motor and sensory function[42]. This finding suggests that different inhibitors, or even the same inhibitors of different lots, can lead to completely contradictory experimental results. The possible reasons discussed above are merely speculations, and the exact causes still needs to be determined by further experiments.

In order to elucidate the mechanism through which pEGFR promotes the recovery of neural functions in rats with CSCI, we further evaluated the expression of pAkt1 (a downstream signal molecules of pEGFR). This enzyme belongs to the AKT subfamily of serine/threonine kinases that contain SH2 (Src homology 2-like) domains, and can be activated by pEGFR through the phosphotyrosine-binding SH2 domains. Previous reports indicated that pAkt1 can not only up-regulate the expression of Olig2 to promote the proliferation and differentiation of OPCs, but also down-regulate the expression of caspase family to inhibit the apoptosis of OLs[43–45]. Hence, we hypothesize that after decompression of CSCI, pEGFR promotes the proliferation and differentiation of OPCs by activating pAkt1, thereby enhancing the recovery of nerve functions. The results illustrated in Figs. 3–6 are in support of this hypothesis. Taken together, pEGFR has been shown to promote functional recovery of rats with CSCI by activating pAkt1.

We only performed experiments in vivo, and future research should include in vitro studies and explore the effects of different microenvironments on the proliferation and differentiation of OPCs.
Conclusions

In conclusion, pEGFR promotes the recovery of neural function in rats after decompression of CSCI. This effect is achieved by activation of pAkt1 and the consequent promotion of the proliferation and differentiation of OPCs. This study provides an experimental and theoretical basis for molecularly-targeted therapy of the complications after decompression of CSCI.

Declarations

Acknowledgements

Not applicable.

Abbreviations

BBB Basso, Beattie & Bresnahan
CSCI compressed spinal cord injury
LFB luxol fast blue
pEGFR phosphorylated epidermal growth factor receptor
pAkt1 phosphorylated Akt1
OPCs oligodendrocyte precursor cells
HER epidermal growth factor receptor
SD Sprague-Dawley
N normal group
D0 model 0 day group
D1 model 1 day group
D7 model 7 day group
D14 model 14 day group
D21 model 21 day group (D21 intervention group)
OCT optimal cutting temperature
one-way ANOVA one-way analysis of variance
LSD Least—Significant Difference
OLs oligodendrocytes
WB Western Blot

Ethics approval and consent to participate

All experimental procedures were performed in accordance with the Animal Care and Ethics Committee and were approved by the Ministry of Science and Technology of the People’s Republic of China.

Consent to publication
Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors state that there are no actual or potential conflicts of interests.

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Authors’ contributions

Shanquan Sun conceived and designed the experiments. Rui Gong and Min Zhang performed the experiments. Shanquan Sun, Rui Gong and Min Zhang analyzed the data. Shanquan Sun contributed reagents, materials and analysis tools. Rui Gong and Min Zhang wrote the paper. Kexin Sun, Wei Qi and Wentao Zhen revised the article critically for important intellectual content. Zhong Yuan and Jianjun Li are responsible for polishing the language of the article. All the authors agree to be accountable of all aspects of the work. All authors read and approved the final manuscript.

Submission declaration and verification

The authors have not published or submitted the manuscript elsewhere.

References


Figures

Figure 1 Neurological function assessment. BBB scores assessed based on the Basso, Beattie, and Bresnahan (BBB) rating scale. Data represent mean±SD (n = 20 per group). One way ANOVA was performed followed by LSD post hoc tests where appropriate. # P < 0.05, compared with the normal group; &P < 0.05, compared with model group; *P < 0.05, compared with the intervention group.
Figure 2 Myelinated nerve fibers. Myelinated fibers were counted by blinded investigator using Image-J software. Data represent mean±SEM (n = 12 per group). One-way ANOVA was performed followed by LSD post hoc tests where appropriate. # P < 0.05, compared with the normal group; & P < 0.05, D14 vs. D0; * P < 0.05, compared with the intervention group.

Figure 2
Figure 3 pEGFR protein expression at the corresponding time examined by Western blot. One-way ANOVA was performed followed by LSD post hoc tests where appropriate. The expression of pEGFR was low in N, D0 and D1. Compared with N, D0 and D1, the expression of pEGFR was significantly increased in D7, D14 and reached the peak at D14. Two independent samples t-test was performed: The expression of pEGFR in the intervention group was significantly lower than that in the 14 day group. Data represent mean±SD (n = 3 per group). # P < 0.05, D7 vs. N, D7 vs. D0, D7 vs. D1, D14 vs. N, D14 vs. D0, D14 vs. D1; &P < 0.05, D7 vs. D1, D14 vs. D7; *P < 0.05, D14 vs. I.
Figure 4 Double-labeling immunofluorescence of pEGFR and NG2, an oligodendrocyte precursor cells marker, in the white matter of the spinal cord of rats in different groups. The pEGFR-NG2+ cells were scattered in the white matter in N, D0 and I, while the density of pEGFR-NG2+ cells increased significantly in D14. One-way ANOVA was performed followed by LSD post hoc tests where appropriate. P<0.05, D14 group vs. N group, D0 group or I group.
Figure 5 pAkt1 protein expression at the corresponding time examined by Western blot. One-way ANOVA was performed followed by LSD post hoc tests where appropriate. The expression of pAkt1 was low in N, D0 and D1. Compared with N, D0 and D1, the expression of pAkt1 was significantly increased in D7, D14 and reached the peak at D14. Two independent samples t-test was performed: The expression of pAkt1 in the intervention group was significantly lower than that in the 14 day group. Data represent mean±SD (n = 3 per group). *P < 0.05, D7 vs. N, D7 vs. D0, D7 vs. D1, D14 vs. N, D14 vs. D0, D14 vs. D1; &P < 0.05, D7 vs. D1, D14 vs. D7; *P < 0.05, D14 vs. I.
Figure 6 Double-labeling immunofluorescence of pAkt1 and NG2, an oligodendrocyte precursor cells marker, in the white matter of the spinal cord of rats in different groups. The pAkt1-NG2+ cells were scattered in the white matter in N, D0 and I, while the density of pEGFR-NG2+ cells increased significantly in D14. One-way ANOVA was performed followed by LSD post hoc tests where appropriate. P<0.05, D14 group vs. N group, D0 group or I group.

Figure 6

Supplementary Files

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