NEURAL REGENERATION RESEARCH
Volume 7, Issue 11, April 2012
www.nrronline.org

Cite this article as: Neural Regen Res. 2012;7(11):810-814.

Telomerase expression in the glial scar of rats with spinal cord injury★

Mingkun Yang[1], Weibin Sheng[1], Tao Xu[1], Kai Huang[1], Yanjiao Wang[2]

1*Department of Spinal Surgery, First Affiliated Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China*
2*Department of Molecular Biology, Basic Medical College, Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China*

Abstract

A rat model of spinal cord injury was established using the weight drop method. A cavity formed 14 days following spinal cord injury, and compact scar tissue formed by 56 days. Enzyme-linked immunosorbent assay and polymerase chain reaction enzyme-linked immunosorbent assay results demonstrated that glial fibrillary acidic protein and telomerase expression increased gradually after injury, peaked at 28 days, and then gradually decreased. Spearman rank correlation showed a positive correlation between glial fibrillary acidic protein expression and telomerase expression in the glial scar. These results suggest that telomerase promotes glial scar formation.

Key Words: spinal cord injury; glial scar; telomerase; astrocytes; glial fibrillary acidic protein

Abbreviations: SCI, spinal cord injury; GFAP, glial fibrillary acidic protein; NF-200, neurofilament-200

Mingkun Yang★, Studying for master's degree, Department of Spinal Surgery, First Affiliated Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China

Corresponding author: Weibin Sheng, M.D., Chief physician, Professor, Department of Spinal Surgery, First Affiliated Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China
wbsheng@vip.sina.com

Received: 2011-12-27
Accepted: 2012-03-15
(N20111119003/YJ)

Yang MK, Sheng WB, Xu T, Huang K, Wang YJ. Telomerase expression in the glial scar of rats with spinal cord injury. Neural Regen Res. 2012;7(11):810-814.

www.crter.cn
www.nrronline.org

doi:10.3969/j.issn.1673-5374.2012.11.002

INTRODUCTION

Telomerase is a ribonucleoprotein that compensates for the shortening of the ends of linear DNA by adding telomeric repeats onto the ends of chromosomes by using an integral RNA as the template. While telomerase expression is suppressed in most normal tissues, it is over-expressed in tumors and scar tissue[1]. Repetitive DNA fragments are added to telomeres to compensate for replication-induced telomere shortening, resulting in rapid cell activation and prolonged life span[2].

The tissue response to spinal cord injury (SCI), including glial scar formation, appears to be associated with the induction of telomerase expression[3]. After SCI, glial fibrillary acidic protein (GFAP) expression is increased in astrocytes. In addition to being a marker of astrocyte activation, GFAP can be used to assess glial scar formation[4]. In the present study, we measured telomerase expression during glial scar formation using telomerase polymerase chain reaction (PCR) enzyme linked immunosorbent assay (PCR ELISA). We also analyzed changes in GFAP expression, and investigated the relationship between telomerase expression and glial scar formation.

RESULTS

Quantitative analysis of experimental animals

A total of 80 Sprague-Dawley rats were equally and randomly divided into model and control groups. The SCI model was established using a modified Allen's weight drop method. Two rats died during surgery, and three died after surgery. Dead rats were supplemented by new rats. Infection did not occur after surgery. A total of 80 rats were included in the final analysis.

Motor function changes in rats with SCI

Scores for the Basso, Beattie and Bresnahan (BBB) locomotor rating scale were similar between model and control groups before surgery ($P > 0.05$). In the model group, hindlimb function disappeared 1 day after SCI (BBB score: 0), gradually recovered, and reached a plateau phase at 21–28 days. BBB scores in the model group were significantly lower compared with the control group at various time points following surgery ($P < 0.01$; Figure 1).

Morphologic changes in the spinal cord after injury

Hematoxylin-eosin staining demonstrated that axons were arranged in parallel bundles in the spinal cord of rats from the control group (Figure 2A). In the model group, edema, hemorrhaging, neuronal swelling and necrosis were visible 1 day after surgery, and a hollow cavity was detectable in the injured region at 14–28 days (Figure 2B). Compact scar tissue formed at the site of injury, and a small cavity was observed at 56 days (Figure 2C).

GFAP and neurofilament protein are markers of astrocytes and neurons, respectively[4-5].

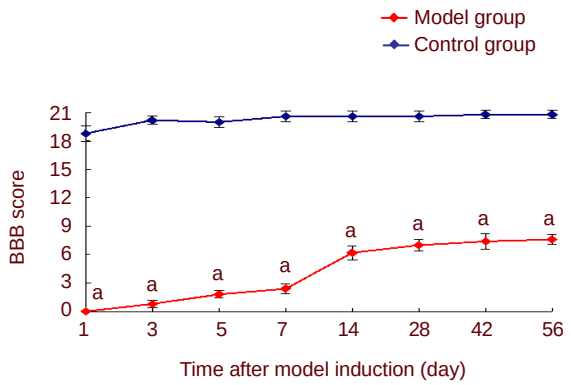

Figure 1 Mobility in rats with spinal cord injury. The higher the Basso, Beattie and Bresnahan (BBB) score, the better the hindlimb function.

Intergroup comparison was done using one-way analysis of variance. Data were expressed as mean ± SD of five rats in each group at various time points. $^{a}P < 0.01$, *vs*. control group.

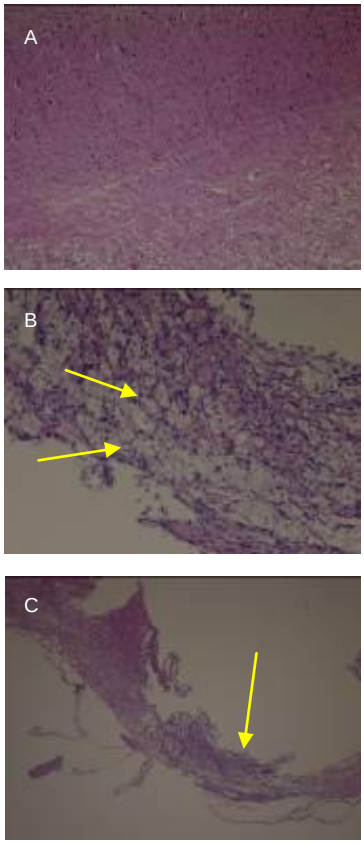

Figure 2 Optical microscopy of the rat spinal cord (hematoxylin-eosin staining, × 100).

(A) Normal spinal cord in rats from the control group.

(B) Hollow cavity (arrows) in the spinal cord at 28 days after injury in the model group.

(C) Scar tissue formation (arrow) in the model group at 56 days after injury.

Immunofluorescence labeling demonstrated normal astrocyte morphology in the grey and white matter of the spinal cord in the control group. In the model group, astrocytes were activated and they had enlarged cell bodies and thickened processes 1–28 days following SCI (Figure 3A), and finally they formed a glial scar.
The number of neurofilament-200 (NF-200)-labeled neurons gradually diminished following SCI, with numbers stabilizing by 28 days (Figure 3B). Nerve fibers were not observed in the glial scar or in the hollow cavity at 56 days.

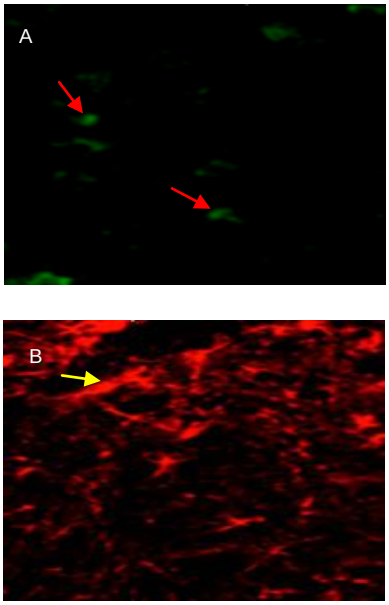

Figure 3 Laser confocal microscopy of glial fibrillary acidic protein (GFAP) (A) and neurofilament-200 (NF-200) (B) expression in the rat spinal cord 28 days following spinal cord injury (immunofluorescence, × 100).

Fluorescent stain was fluorescein isothiocyanate. GFAP-positive astrocytes (green) and NF-200-positive neurons (red) are shown. Arrows represent positive expression.

GFAP expression in the glial scar following SCI

ELISA showed that GFAP expression increased after SCI, peaked at 28 days, and then gradually decreased in the model group. GFAP expression did not significantly change in the control group. GFAP expression was significantly higher in the model group compared with the control group at 1–56 days ($P < 0.01$; Table 1).

Telomerase expression in the glial scar following SCI

Telomerase PCR ELISA results showed that telomerase expression was detectable at 1 day, peaked at 28 days, and then gradually decreased in rats from the model group. Telomerase expression was not determined at various time points in the control group. Telomerase expression was significantly higher in the glial scar of rats from the model group compared with the control group ($P < 0.05$; Table 2).

Table 1 Glial fibrillary acidic protein expression in the glial scar of rats with spinal cord injury (difference value of A_{450nm} to A_{690nm})

Group	Time after injury (day)			
	1	3	5	7
Model	1.98±0.15	6.08±0.23	9.04±0.35	12.30±0.45
Control	0.46±0.05	0.48±0.04	0.46±0.05	0.46±0.05
t	21.496	53.886	54.049	58.761
P	0.000	0.000	0.000	0.000

Group	Time after injury (day)			
	14	28	42	56
Model	17.50±0.50	19.40±0.55	16.60±1.14	12.60±1.14
Control	0.46±0.05	0.46±0.05	0.44±0.05	0.48±0.04
t	75.752	76.938	31.656	23.751
P	0.000	0.000	0.000	0.000

Intergroup comparison was done using one-way analysis of variance. Data were expressed as mean ± SD of five rats in each group at various time points.

Table 2 Telomerase expression in glial scar of rats with spinal cord injury (difference value of A_{450nm} to A_{690nm})

Group	Time after injury (day)			
	1	3	5	7
Model	0.274±0.005	0.386±0.004	0.435±0.005	0.557±0.006
Control	0.072±0.007	0.073±0.006	0.072±0.008	0.074±0.005
t	51.813	97.276	89.320	137.776
P	0.000	0.000	0.000	0.000

Group	Time after injury (day)			
	14	28	42	56
Model	0.757±0.021	1.217±0.072	0.660±0.011	0.180±0.004
Control	0.075±0.004	0.074±0.005	0.073±0.006	0.074±0.006
t	72.030	35.626	103.068	30.663
P	0.000	0.000	0.000	0.000

Intergroup comparison was done using one-way analysis of variance. Data were expressed as mean ± SD of five rats in each group at various time points.

Spearman rank correlation demonstrated that telomerase expression was positively associated with GFAP expression in the glial scar of rats with SCI (r = 0.755, P < 0.01; Figure 4).

DISCUSSION

GFAP, an intermediate filament protein, is a major component of the astrocyte cytoskeleton and is required for the morphology and function of astrocytes[4]. In the present study, we observed proliferation and hypertrophy of astrocytes and increased expression of GFAP, which is consistent with previous studies[5-6]. GFAP expression was detectable immediately after SCI, gradually diminished by 28 days, and stabilized by 56 days. Neurofilament proteins, part of the cytoskeletal network, play an important role in axoplasmic transport and neuronal survival. Changes in neurofilament expression are strongly correlated with pathophysiological changes in the spinal cord[7]. Our study showed that NF-200 expression was not significantly altered in the glial scar in the spinal cord.

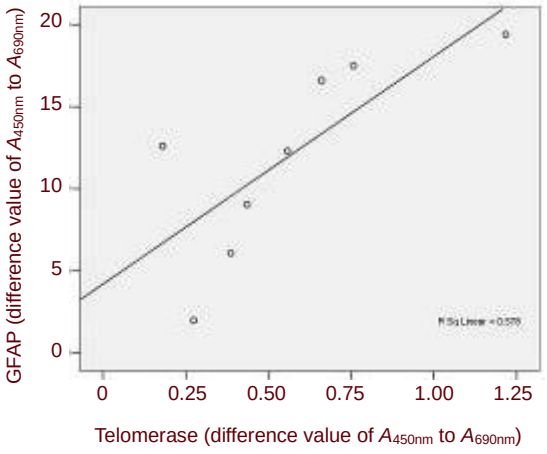

Figure 4 Relationship between telomerase expression and glial fibrillary acidic protein (GFAP) expression in the glial scar of rats with spinal cord injury. Spearman rank correlation revealed a positive correlation between telomerase and GFAP expression (r = 0.755, P < 0.01).

Telomerase PCR-ELISA was used to assess dynamic changes in telomerase expression in the glial scar. We found that telomerase expression was weakly detected at 1 day, and reached a peak at 28 days. Results from Spearman rank correlation demonstrated that GFAP expression was positively correlated with telomerase expression in the glial scar, suggesting that telomerase may be associated with glial scar formation.

In summary, telomerase expression exhibited a linear relationship with glial scar formation. Telomerase expression contributed to astrocyte activation, resulting in the formation and maintenance of the glial scar.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

Experiments were performed at the Animal Center and Molecular Biology Laboratory, Xinjiang Medical University, China, from February to November 2011.

Materials

A total of 80 clean male Sprague-Dawley rats weighing 180–220 g were supplied by the Animal Experimental Center, Xinjiang Medical University (license No. SYXK (Xin) 2003-001). Protocols were conducted in accordance with the *Guidance Suggestions for the Care*

and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[8].

Methods

Establishment of the SCI model

The rats were intraperitoneally injected with a 0.5 mL/100 g anesthetic solution (2 mL ketamine, 1 mL atropine, 2 mL diazepam, 5 mL 0.85% sodium chloride). In the prone position, a 2 cm-long incision was made, and T_{10} was taken as the center. T_9 and T_{11} interspinal ligaments were cut to dissociate muscles beside the spinous process. After removal of the $T_{9\text{-}11}$ spinous processes and vertebral plate, a circular area of 4.5 mm diameter was exposed with T_{10} at the center. Using modified Allen's weight drop method[9], the dorsal surface of the spinal cord at T_{10} was impacted with a 30 g weight dropped from a height of 5 cm. Rapid hindlimb contraction and intense tail movement represented successful model establishment. The bladder was manually squeezed to help the rats to urinate every 8 hours after the surgery until automatic micturition recovered. The spinal cord was exposed, but not injured, in the control group.

BBB scoring of rat motor function

Five rats were taken from each group 1 day before surgery, and 1–7, 14, 28, 41 and 56 days after surgery for BBB scoring (scores 0–21)[10]. The higher score represented better recovery of motor function.

Sample collection

Five rats were obtained from each group at 1, 3, 5, 7, 14, 28, 42 and 56 days after model establishment. The rats were sacrificed using the depletion method[11]. 50-mg samples of spinal cord tissue were collected from the injured region, washed in diethyl pyrocarbonate solution, and stored in an RNase-free tube at −80°C.

Hematoxylin-eosin staining

The specimens were fixed and stored in 30% sucrose overnight at 4°C, and then serially sliced into 4-μm thick sagittal sections. These sections were rinsed in distilled water for 2 minutes, stained in hematoxylin (Zhongshan Biotechnology, Beijing, China) for 3–5 minutes, washed in running water for 10 minutes, rinsed in distilled water for 5 seconds, treated with 95% alcohol for 5 seconds, stained in eosin (Zhongshan Biotechnology) for 1 minute, dehydrated in gradient alcohol, permeabilized with xylene, mounted in neutral gel, and observed under an optical microscope (Olympus, Tokyo, Japan).

Immunofluorescence labeling for GFAP and NF-200 expression

Frozen sections were washed three times in phosphate buffered saline (PBS), each 10 minutes, blocked in 10% goat serum at room temperature for 1 hour, followed by incubation in rabbit anti-rat GFAP and NF-200 antibodies (1:100, Sigma, St. Louis, MO, USA) at 4°C overnight. Following three washes in PBS (10 minutes each), the sections were incubated in fluorescein isothiocyanate-labeled goat anti-rabbit antibody (1:100, Sigma) at room temperature for 2 hours. After three washes in PBS, sections were mounted in a fluorescent sealer, and observed using a laser confocal microscope (Olympus).

GFAP expression in glial scar of the spinal cord, as determined by enzyme linked immunosorbent assay

Protein was extracted from glial scar in accordance with instructions of the kit. The specimens were coated with a microplate sealer at 37°C for 30 minutes. The fluid was removed, and specimens were dried. Cleaning solution was added in each well and was discarded 30 seconds later. This procedure was repeated for five times. The specimens were patted dry, incubated in 50 μL assay reagent at 37°C for 30 minutes. After removal of reagent, cleaning solution was added for 30 seconds. A total of 50 μL developer A was added in each well, and then 50 μL developer B was added, slightly shake, mixed, and developed in the dark at 37°C for 15 minutes. The reaction was terminated by 50 μL stop buffer. Absorbance in each well was measured at 450 nm. The measurement was repeated three times in each specimen, and the mean value served as final results.

PCR ELISA detection of telomerase expression in the glial scar

The glial scar of the spinal cord was washed twice in PBS, treated with 200 μL protein extraction buffer, placed on ice for 30 minutes, and centrifuged. 175 μL of supernatant was transferred into an Eppendorf tube. 25 μL PCR reaction reagent (biotin labeled forward oligonucleotide primer pl-TS, reverse primer p2, Taq polymerase; Roche, Nutley, NJ, USA) and 2 μL protein extract were added to the tube. Total volume was 50 μL after adding diethyl pyrocarbonate solution. The tube was placed in a PCR instrument (Roche) for primer extension/amplification reaction. Themocycling was as follows: extension at 25°C for 30 minutes, inactivation at 94°C for 5 minutes; amplification conditions (30 cycles): denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and PCR at 72°C for 90 seconds. 20 μL denaturation solution and 5 μL of the amplified product were transferred to a centrifuge tube and incubated at 15–25°C for 10 minutes. Hybridization buffer was added to each centrifuge tube and the tube was placed on an oscillating shaker. 225 μL hybridization solution containing digoxin-labeled telomeric sequence-specific probe was added to each microplate well. After shaking for 2 hours at 37°C in the dark, hybridization solution was discarded, and anti-DIG-POD working fluid (Roche) was added. Following shaking for 30 minutes in the dark at room temperature, the solution was discarded, and TMB substrate solution was added. After shaking at room temperature for 20 minutes in the dark, stop buffer was added. Absorbance values were measured at 450 and 630 nm using a microplate reader (BioRad). $Absorbance_{450nm}-Absorbance_{690nm} < 0.1$ represented negative expression of telomerase; values > 0.2 represented positive expression; values between 0.1 and 0.2 represented false positive expression, and required detection again. Each specimen was examined three times, and the mean value was taken as the final result.

Statistical analysis

The data were expressed as mean ± SD, and analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). BBB scores, and telomerase and GFAP expression were compared among groups utilizing one-way analysis of variance. Correlation analysis of telomerase and GFAP expression was done using Spearman rank correlation. A value of $P < 0.05$ was considered statistically significant.

Acknowledgments: We thank Animal Experimental Center, Xinjiang Medical University in China for providing the experimental platform and the Department of Molecular Biology, Xinjiang Medical University in China for technical guidance.
Funding: This project was funded by the National Natural Science Foundation of China, No. 81060106.
Author contributions: Mingkun Yang participated in study design and implementation and wrote the manuscript. Tao Xu, Kai Huang and Yanjiao Wang were responsible for study evaluation. Mingkun Yang, Tao Xu, Kai Huang and Yanjiao Wang were in charge of data collection. Weibin Sheng was in charge of manuscript authorization.
Conflicts of interest: None declared.
Ethical approval: This study was approved by the Animal Ethics Committee, Xinjiang Medical University, China.

REFERENCES

[1] Wu A, Ichihashi M, Ueda M. Correlation of the expression of human telomerase subunits with telomerase activity in normal skin and skin tumors. Cancer. 1999;86(10):2038-2044.
[2] Allsopp RC, Chang E, Kashefi-Aazam M, et al. Telomere shortening is associated with cell division in vitro and in vivo. Exp Cell Res. 1995;220(1):194-200.
[3] Basso DM, Beattie MS, Bresnahan JC. Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. Exp Neurol. 1996;139(2): 244-256.
[4] Takizawa T, Gudla PR, Guo L, et al. Allele-specific nuclear positioning of the monoallelically expressed astrocyte marker GFAP. Genes Dev. 2008;22(4):489-498.
[5] Eng LF, Ghirnikar RS. GFAP and astrogliosis. Brain Pathol. 1994; 4(3):229-237.
[6] Uchida K, Baba H, Maezawa Y, et al. Progressive changes in neurofilament proteins and growth-associated protein-43 immunoreactivities at the site of cervical spinal cord compression in spinal hyperostotic mice. Spine (Phila Pa 1976). 2002;27(5): 480-486.
[7] Duan YP, Wang JD, Huang YQ, et al. The changes of neurofilament 200 expression after GAP-43 therapy on acute complete spinal cord injury rats. Shenjing Jiepou Xue Zazhi. 2008; 24(4):406-410.
[8] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.
[9] Allen AR. Surgery of experimental lesions of spinal cord equivalent to crush injury of fracture dislocation. JAMA. 1911; 57(5):878-880.
[10] Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma. 1995;12(1):1-21.
[11] Li FK, Wang CY. Experimental Animals and Animal Experiments Methodology. Zhengzhou: Zhengzhou University Press. 2007.

(Edited by Gu P, Du JR/Qiu Y/Song LP)