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ORIGINAL ARTICLE

The relationship between macrophage polarization and glial scar formation in mouse model of spinal cord injury

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Abstract

OBJECTIVE: The study aimed to investigate the relationship between macrophage polarization and glial scar formation in mice model of spinal cord injury (SCI).
METHODS: A total of 40 specific pathogen-free male C57BL/6 mice were randomly divided into the model (n=20) and control (n=20) groups. The model group was divided into 1-d, 7-d, 14-d, and 28-d post-model groups, with 5 mice in each group. SCI at T9-10 levels was produced by freely dropping a 10 g weight from a height of 5 cm onto the T9-10 spinal segment. The control group underwent the same procedures without damaging the spinal cord. Spinal cord tissue samples were obtained at 1d, 7d, 14d, and 28d after SCI, HE and immunohistochemical staining were used to observe glial scar formation following SCI. RT-qPCR and ELISA assay were used to detect the expression of M1 markers TNF-a, IL-1β, and M2 markers Arginase-1, IL-10.
RESULTS: HE and immunohistochemical staining showed glial scar formation in the model group, while no glial scar formation was observed in the control group. Results from RT-qPCR and ELISA showed that the expression of IL-1β and TNF-α in the model group were significantly higher compared with the control group at each time point (both P <0.01). Highest expression of IL-1β and TNF-α was observed on days 7, which gradually decreased, and remained stable on day 28 day after SCI in the model group. No significant change in IL-1β and TNF-α expresseion was obsreved in the control groups. the expression of IL-10 and Arginase-1 in the model group were significantly higher compared with the control group at each time point (both p <0.01). IL-10 and Arginase-1 expression reached its maximum level on day 14, then gradually decreased, and remained stable on day 28 day after SCI in the model group. No significant change in IL-10 and Arginase-1expression was observed in the control group at each time point.
CONCLUSIONS: Macrophages were are mainly polarized to M1 phenotype in the first 7 days during glia scar formation after SCI, which were then gradually polarized into M2 phenotype at 7 days, and tended to be stabilized at 28 days after SCI.

INTRODUCTION

Spinal cord injury (SCI) is a common event, complete SCI is often complicated with many other diseases, and even leads to death (Bárbara-Bataller *et al.* 2017). At present, the treatment options for SCI and their efficacy are still limited. The reason is that the formation of glial scar after SCI hinders axon repair, and glial scar formation is a complex pathological process (Kuhlmann *et al.* 2017; Hara *et al.* 2017; Kuhlmann *et al.* 2017), which inhibits nerve regeneration. The pathological process of SCI can be classified into primary and secondary injury process (David *et al.* 2012; Sun *et al.* 2013). The area and severity of secondary injury after primary injury are far greater than the primary injury (Francos-Quijorna *et al.* 2017), glial scar formation mainly occurs in the secondary injury process after SCI. The main causes of secondary injury include the excitotoxicity of damaged cells in the injured area, the formation of free radicals, disruption of blood vessels and inflammatory response (Francos-Quijorna *et al.* 2017; Ray *et al.* 2003; Smith *et al.* 2010). Inflammatory response is the main cause of glial scar formation, which is crucial for the prognosis of SCI (Vidal *et al.* 2018).

Inflammation is one of the most important pathological processes following central nervous system (CNS) injury. Macrophage is the most important inflammatory cell in the inflammatory response, which has a dual role, i.e. aggravating the inflammatory response or promoting tissue regeneration (Shechter *et al.* 2013; Nguyen *et al.* 2017; Shechter *et al.* 2013). The dual role of macrophages is associated with the different macrophages polarization phenotypes after SCI (Shimbo *et al.* 2017; Wattananit *et al.* 2016; Haan *et al.* 2015). However, the relationship between macrophage polarization and glial scar formation have not yet been reported. Therefore, the present study aimed to explore how macrophages are polarized during the formation of glial scars, as well as their relationships, and provided new ideas for promoting the treatment of SCI by regulating the polarization of macrophages.

MATERIALS AND METHODS

Experimental animal grouping

A total of 40 specific pathogen-free male C57BL/6 mice (45-56 days of age) were purchased from China three gorges university, and were randomly divided into model and control groups, with 20 mice in each group. Rats in model group were divided into 1-d, 7-d, 14-d, and 28-d post-model groups, with 5 mice in each group. All experimental procedures were approved by ethics committee of our hospital.

Establishment of mice model of SCI

After 3 days of acclimation, all mice were anesthetized intraperitoneally with 4% chloral hydrate. After hair removal and skin disinfection in the surgical field, and a longitudinal incision was made centered on T10 spinous process, then the T9, T10 spinous processes and lamina were removed to expose the dura mater. In the model group, SCI at T9-10 levels was produced by freely dropping a 10 g weight from a height of 5 cm using a weight drop device onto the T9-10 spinal segment (Chen *et al.* 2014). The signs of successful model establishment included spastic swinging of the tail and retraction-like flutter and flaccid paralysis of the hind limbs. In the control group, T9-10 spinous processes and lamina were removed without damaging the spinal cord. After surgery, the mice were kept warm. And after recovery from anaesthesia, mice were moved to the animal room. All mice were fed normally. 20,000 units of penicillin were injected intraperitoneally into mice once a day after surgery, and the bladder of the mice was squeezed manually twice daily. If mice died during surgery, new mice was added and animal model was established again.

Observation of glial scar formation

At 1d, 7d, 14d, and 28d after SCI, spinal cord tissue samples were obtained from the injured area of each mouse in the model group, HE staining and immunofluorescence staining were performed to observe glial scar formation.

Hematoxylin-eosin (HE) staining

Samples were dehydrated by 75%, 85%, 90%, 95% ethanol and absolute ethanol, then the samples were cleared, impregnated with wax, embedded, sectioned, baked, dewaxed, and stained. The sections were stained with Mayer's hematoxylin for 5min, then washed with tap water until they were blue. Then the sections were rinsed in 1% hydrochloric acid alcohol for 2-5 seconds, followed by staining with 1% water soluble eosin solution for 5min, the sections were washed with tap water for 30s, air dried, sealed with neutral balsam and finally observed under a microscope.

Immunohistochemical staining

After the samples were dehydrated with 75%, 85%, 90%, 95% ethanol and absolute ethanol, cleared, impregnated with wax, embedded, sectioned, baked, and dewaxed, the samples were subjected to antigen retrieval. Endogenous peroxidase activity was blocked. After blocking with serum, the sections were incubated with primary antibody, enzyme-labeled secondary antibody. After adding chromogenic reagent, the sections were counterstained, dehydrated and sealed, the sealed sections were placed in a fume hood until they were dried, and then observed under a microscope.

Detection of macrophage markers using real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using the Trizol reagent (Thermo Fisher Scientific, Inc. USA). Reverse transcription for DNA was synthesized using PrimeScript™

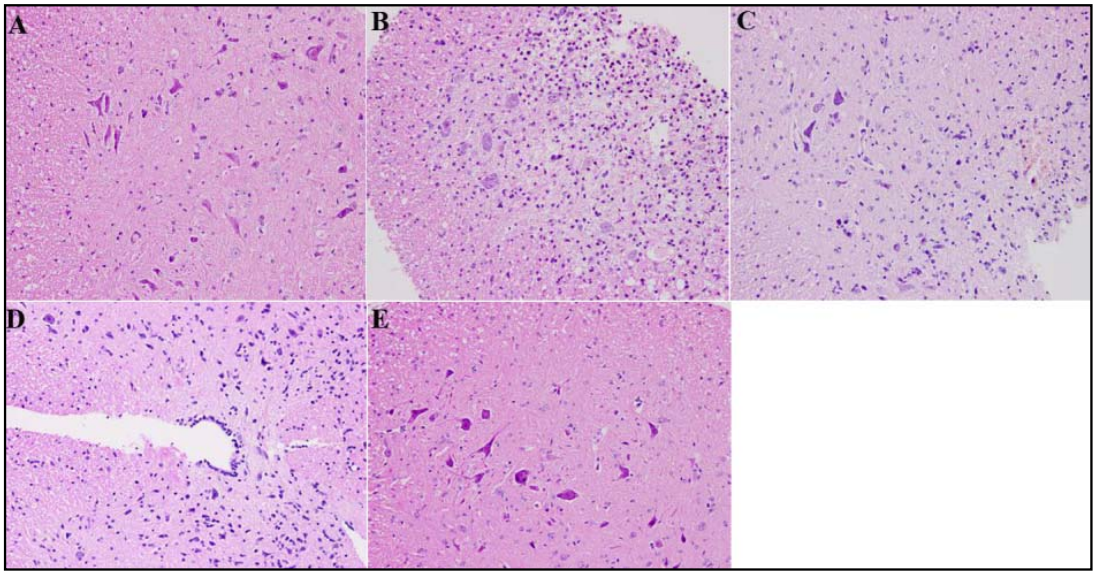

Fig. 1. HE staining (×200 magnification) of spinal cord tissue in mice of model group on day 1 (A), day 7 (B), day 14 (C), day 28 (D) after injury, and control group (E).

reverse transcription kit (Takara, Japan). RT-qPCR was performed in a 10 μL reaction system using UltraSYBR premixture (CWbio Co., Ltd., Beijing, China). RT-qPCR conditions included 10 min of predenaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing/ elongation at 60 °C for 1 min. The dissolution curve conditions were 95°C for 15 S, 60°C for 1 min, 95°C for 15 s, 60°C for 15 s. The relative quantification was calculated using the 2−ΔΔCT method with reference genes β-actin.

Dectection of macrophage markers using Enzyme-linked immunosorbent assay (ELISA)

ELISA was perfromed according to the manufacturer's instrucns, samples were added to wells, the paltes were sealed with a sealing film and incubated at 37°C for 30 minutes; then the sealing film was carefully removed, the liquid was discarded, after spin drying, and filling each well with wash buffer, and wash buffer was discarded after resting for 30 seconds. After repeated washing 5 times, plates were pat dried, 50 μL of enzyme labeled was added to each well, except the blank well, and incubated at 37°C for 30 min, then the liquid was discarded. After spin drying, and filling each well with wash buffer, wash buffer was discarded after resting for 30 seconds. After repeated washing 5 times, plates were pat dried, 50μl substrater A, and 50μl substrate B were added to each well, mixed with gently shaking and incubated at 37°C for 15 minutes in dark. Then 50μl stop solution was added to each well to stop the reaction. The OD value of each well was measured at wavelength of 450 nm.

Statistical analysis

Statistical analysis was performed using SPSS 23.0 software, and measurement data was expressed mean±SD. Chi-square test was used for the comparison between groups. Least significant difference (LSD) test was used to compare the differences in the indicators among groups. Correlation analysis was performed by Spearsman rank correlation test. A one-sided *P*-values <0.05 were considered statistically significant.

RESULTS

Observation of glial scar formation

HE staining results showed that bleeding in the model group was obvious at day 1 after SCI. The motor neurons of the anterior horn were swollen and inflammatory cells gathered. And more neurons died and astrocytes were activated and proliferated at 7-14 days after injury. These phenomenons were not observed in the control group (Figure 1).

After immunohistochemical staining, image-Pro Plus 6.0 software was used to observed the positively staining cells (dark brown). The results showed that the number of cells stained dark brown accounted for 95.0% of the total cells in the model group, while the cell morphology in the control group was normal (Figure 2).

Expression of macrophage markers detected by RT-qPCR

The results showed that the expression of M1-type markers, IL-1β and TNF-α in the model group were significantly higher compared with the control group at each time point after SCI (X^2=36.75-85.42, P<0.01). Highest expression of IL-1β and TNF-α was observed at 7 days after SCI, then gradually decreased, and remained stable at day 28 after SCI in model group. No significant change in the expression of TNF-a and IL-1β was observed in the control group (P>0.5). The expression of M2-type markers, Arginase-1 and

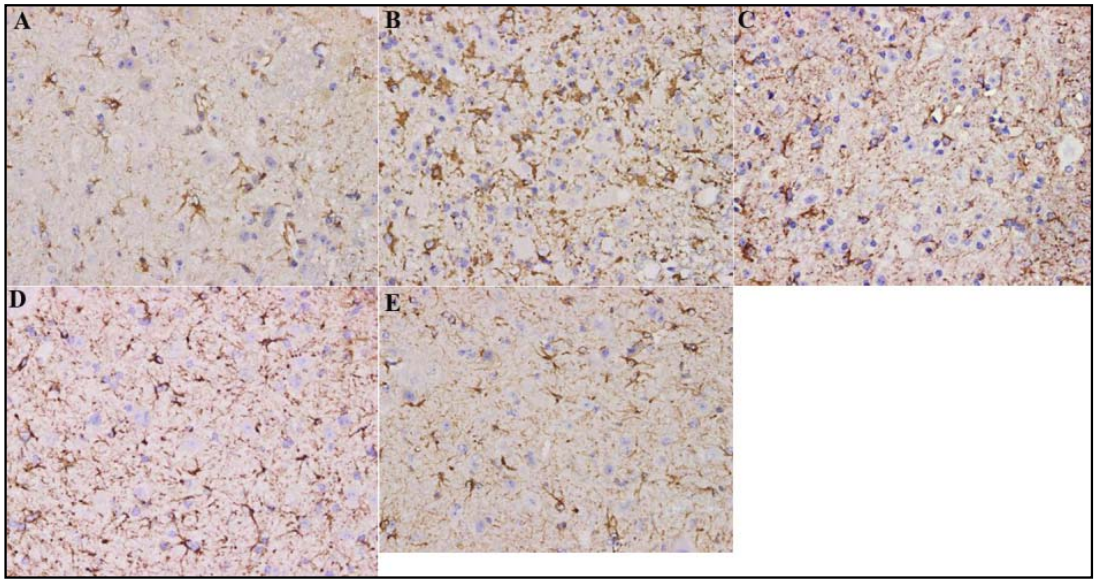

Fig. 2. Immunohistochemical staining (×400 magnification) of spinal cord tissue in mice of model group on day 1 (A), day 7 (B), day 14 (C), day 28 (D) after injury, and control group (E).

IL-10 in the model group were significantly higher compared with the control group at each time point after SCI (X^2=39.58-94.11, P<0.01). Highest expression of Arginase-1 and IL-10 was observed at14 days after SCI, which then gradually decreased, and remained stable at day 28 after SCI in model group. No significant change in the expression of Arginase-1 and IL-10 was observed in the control group at each time point (Table 1).

Expression of macrophage markers detected by ELISA assay

ELISA assay results showed that the expression of M1 markers IL-1β and TNF-α in the model group were significantly higher compared with the control group at each time point (X^2=77.85-368.62, P<0.01). Highest expression of IL-1β and TNF-α was observed at day 7, which then gradually decreased and remained stable at day 28 after SCI in the model group. No significant change in the expression of IL-1β and TNF-α was observed in the control group at each time point (P>0.5). The expression of M2 markers IL-10 and Arginase-1in the model group was significantly higher than that in the control group at each time point (X^2=168.82 -2119.87, P<0.01). IL-10 and Arginase-1 expression reached its maximum level on day 14, then gradually decreased, and remained stable on day 28 day after SCI in the model group. No significant change in IL-10 and Arginase-1expression was observed in the control group at each time point (P>0.5, Table 2).

The relationship between macrophage polarization and glial scar formation

The above mentioned results revealed that scar formation stabilized at 28 days after SCI in the model group. With glial scar formation, the expression of IL-1, IL-1β and TNF-α was increased and peaked at 7 days post sugery, while Arginase-1 and IL-10 expression peaked at 14 days post sugery, which both stabilized at 28 days after injury. These results indicated that macrophages were mainly polarized to M1 phenotype in the first 7 days after SCI, and then gradually polarized into M2 phenotype after 7 days of SCI, which tended to be stabilized at 28 days after SCI.

Tab. 1. Expreseion of macrophage markers detected by RT-qPCR

Time point following surgery	Model group				Control group			
	IL-1β	TNF-α	IL-10	Arginase-1	IL-1β	TNF-α	IL-10	Arginase-1
1d	1.75±0.06	0.81±0.05	1.44±0.02	1.35±0.04	1.30±0.01	0.95±0.02	1.05±0.02	0.92±0.03
7d	3.05±0.04	1.65±0.04	2.03±0.05	1.96±0.02	1.35±0.03	0.69±0.01	0.85±0.01	1.31±0.02
14d	2.05±0.12	1.20±0.05	3.61±0.08	2.72±0.06	0.84±0.03	0.75±0.02	1.04±0.03	1.05±0.03
28d	1.85±0.04	0.94±0.04	2.15±0.05	1.63±0.04	0.67±0.02	0.85±0.03	0.95±0.02	1.06±0.03
F value	71.15	53.75	68.16	64.05	27.45	24.34	29.32	22.51
P value	0.000	0.002	0.000	0.000	0.65	0.85	0.88	0.68

Tab. 2. Expression of macrophage markers detected by ELISA assay (pg/ml)

Time point following surgery	Model group				Control group			
	IL-1β	TNF-α	IL-10	Arginase-1	IL-1β	TNF-α	IL-10	Arginase-1
1d	49.30±2.29	325.65±27.97	66.45±5.69	84.35±4.86	12.30±1.45	78.39±10.54	22.22±1.76	23.35±4.04
7d	62.93±3.81	431.36±34.10	85.98±0.023.94	108.41±3.00	13.21±0.95	67.20±12.07	21.54±1.64	22.65±3.17
14d	37.47±2.81	240.31±27.94	113.489±6.57	136.52±7.70	12.81±1.36	71.06±13.48	24.01±1.55	22.37±2.54
28d	24.95±1.64	169.375±19.07	66.40±6.62	88.58±4.14	13.52±0.78	76.68±10.81	23.12±1.66	21.35±2.93
F value	625.06	1839.25	854.85	118.06	265.10	945.01	317.62	387.66
P value	0.000	0.000	0.000	0.000	0.67	0.73	0.75	0.68

DISCUSSION

At present, clinicians face many challenges in the treatment of SCI. Although great progress has been made in cell transplantation treatments for SCI in recent years (Kuhlmann *et al.* 2017; Hara *et al.* 2017), but the problem in the recovery of function following SCI has not been completely solved. The main reason is that the cause of glial scar formation is not fully elucidated. Therefore, exploring the cause of glial scar formation is critical for the treatment of SCI. With the deepening of basic research, it is found that the formation of glial scars is associated with inflammatory response, inflammatory response is the most important pathological process leading to glial scar formation (Vidal *et al.* 2018). Previous study have found that glial scar formation remained stable at 28 days after SCI in rats (Tao *et al.* 2013), so in the present study, the last time point of observation was 28 days after SCI.

Inflammatory response mainly affects axon regeneration and functional recovery after SCI. The local microenvironment changes rapidly following SCI, including neutrophil infiltration and microglia activation, and the accumulation of monocyte-derived macrophages (Shechter *et al.* 2013; Nguyen *et al.* 2017). Macrophages recognize and eliminate the substances in the damaged area that are not conducive to nerve repair, which can promote tissue regeneration (Shechter *et al.* 2013), create conditions for glial scar formation, reduce or prevent the expansion of secondary injury. In this study, our findings showed that the expression levels of M1 type markers IL-1β and TNF-α gradually increased during the first 7 days, and reached a peak at 7 days post-injury, this is consistent with the increased inflammatory responses during first 7 day post injury, while the expression levels of M-12 markers IL-10 and Arginase-1 gradually increased from 7 to 14 days post injury, and reached a peak at 14 days, this may be linked to gradual decrease in inflammatory responses. Wang et al (2017) established model of traumatic brain injury in mice and found that macrophages isolated from the ipsilateral cerebral cortex were activated with increased expression of NADPH oxidase 2 after 7 days of injury, which can significantly inhibit the growth of neurons and enhance the inflammatory response. This result indicated that macrophage-mediated inflammatory response can also exert inhibitory effects on nerve repair after CNS injury. Therefore, macrophages play a dual role in the repair of SCI. Results from our study and the above mentioned previous studies both suggest that the mechanism underlying this dural role may be associated with the changes in different macrophages polarization phenotypes caused by the changes in the local microenvironment after SCI.

Different patterns of macrophages polarization can be induced by different stimulatory factors and different microenvironment of the injured area, which can secrete different cytokines and chemokines, and exert different roles (Murray and Wynn 2011). Macrophage can be polarized into classically activated (M1) and alternatively activated (M2) macrophages (Schwartz and Svistelnik 2012). A study (Haan *et al.* 2015) found that after SCI, M1 and M2 macrophages coexisted at the injury site. After co-culture of M1, M2 macrophages and spinal cord tissues, the author found that M1 macrophages can promote astrocyte proliferation and chondroitin sulfate proteoglycan secretion, contribute to glial scar formation; M2 macrophages have no role in proliferating astrocytes and promoting glial scar formation. In this study, we found that the expression of M1-type markers, IL-1β and TNF-α, reached a peak at 7 days after SCI, while the expression of M2-type markers, IL-10, Arginase-1, reached a peak at 14 days after injury, suggesting that M1-type and M2-type macrophages play different roles in glial scar formation after SCI. Fan *et al.*(2016) showed that after SCI, M1 macrophages induced apoptosis in reactive astrocytes and promoted glial scar formation through the TLR/MyD88 signaling pathway. M2 macrophages are lagging behind M1 macrophages after SCI. Transplantation of M2 macrophages into the injured spinal cord can significantly promote the growth of axons (Kigerl *et al.* 2009), the underlying mechanism may be that M2-polarized macrophages secrete a large amount of fibrous protein and matrix-related proteins, inhibit macrophage infiltration, inflammatory factor levels and activation of their receptors after SCI, and reduce oxidative stress and secondary SCI, promote endothelial cell

proliferation, angiogenesis and recovery of nerve function after SCI (Jia *et al.* 2012).

Glial scar formation and macrophage polarization complement each other and work together to repair the SCI. Glial scars that formed after SCI are mainly composed of activated and proliferated astrocytes and their secreted chondroitin sulfate proteoglycans (Cregg *et al.* 2014). In the early stages of SCI, glial scar formation can prevent further damage to neurons caused by harmful molecules such as excitatory amino acids, reactive oxygen species and free radicals, and inhibit the expansion of secondary injury (Mao *et al.* 2016), these actions of glial scar formation are the same as the role of macrophages in promoting inflammation and removing local necrotic tissue in the early stage of SCI. In the late stage of SCI, M2 macrophages reduce the secretion of pro-inflammatory factors and increase the secretion of neurotrophic factors through regulation of a series of signaling pathways, which can promote the axonal regeneration of residual neurons and functional recovery (David *et al.* 2015; Orr *et al.* 2018), but with the stabilization of the glial scar formation, residual axons cannot pass through the scar area to promote the functional recovery following SCI. Therefore, how to reduce glial scar formation by regulating macrophage polarization is a direction to promote recovery after SCI.

In summary, this study clarified the relationship between glial scar formation and macrophage polarization after SCI, i.e. M1 macrophages were mainly distributed in the early stage of glial scar formation, while M2 macrophages were mainly distributed in the late stage of glial scar formation. Our findings provided certain scientific evidence for promoting recovery of function after SCI by regulating macrophage polarization. However, our study has some limitations worth noting, glial scar formation is a biochemical process involving multiple factors, and M1- and M2-type markers were both expressed during glial scar formation, which indicate that M1 and M2 macrophages or more other cells are involved in glial scar formation, but we only investigate one factor, i.e. macrophages, in this study, Therefore, further investigation is need to investigate the role of other factors in glial scar formation following SCI.

AUTHOR CONTRIBUTIONS

XD conceived and designed the experiments; MK, XD performed the experiments and wrote the paper; KG, ZL, ML, KH analyzed the data; MK, KG, ZL, ML, KH contributed reagents/materials/analysis tools.

DECLARATION OF COMPETING INTEREST

The authors declare that there are no conflicts of interest.

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