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Cetuximab modified collagen scaffold directs neurogenesis of injury-activated endogenous neural stem cells for acute spinal cord injury repair

Xing Li [a, 1], Yannan Zhao [a, 1], Shixiang Cheng [b, 1], Sufang Han [a], Muya Shu [a], Bing Chen [a], Xuyi Chen [b], Fengwu Tang [b], Nuo Wang [a], Yue Tu [b], Bin Wang [a], Zhifeng Xiao [a, **], Sai Zhang [b, ***], Jianwu Dai [a, *]

[a] State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

[b] Tianjin Key Laboratory of Neurotrauma Repair, The Institute of Traumatic Brain Injury & Neuroscience of Chinese People's Armed Police Force (PAP), The Neurosurgery & Neurology Hospital of the Affiliated Hospital of Logistics University of PAP, Tianjin 300162, China

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ABSTRACT

Studies have shown that endogenous neural stem cells (NSCs) activated by spinal cord injury (SCI) primarily generate astrocytes to form glial scar. The NSCs do not differentiate into neurons because of the adverse microenvironment. In this study, we defined the activation timeline of endogenous NSCs in rats with severe SCI. These injury-activated NSCs then migrated into the lesion site. Cetuximab, an EGFR signaling antagonist, significantly increased neurogenesis in the lesion site. Meanwhile, implanting cetuximab modified linear ordered collagen scaffolds (LOCS) into SCI lesion sites in dogs resulted in neuronal regeneration, including neuronal differentiation, maturation, myelination, and synapse formation. The neuronal regeneration eventually led to a significant locomotion recovery. Furthermore, LOCS implantation could also greatly decrease chondroitin sulfate proteoglycan (CSPG) deposition at the lesion site. These findings suggest that endogenous neurogenesis following acute complete SCI is achievable in species ranging from rodents to large animals via functional scaffold implantation. LOCS-based Cetuximab delivery system has a promising therapeutic effect on activating endogenous neurogenesis, reducing CSPGs deposition and improving motor function recovery.

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1. Introduction

Spinal cord injury (SCI) can lead to permanent loss of sensation and voluntary movement below the lesion level and is one of the most challenging clinical problems [1]. Although the characteristics of NSCs in spinal cord of humans is not well documented, however, it is well known that in animals the endogenous neural stem cells (NSCs) remain quiescent in the intact spinal cord, but are immediately activated after SCI and migrate towards the injured epicenter [2–4]. It is also known that only minimal injury to the spinal cord is needed to induce an endogenous NSC response [5]. However, the majority of proliferated NSCs generate new astrocytes to form a glial scar at the injury site, but few newborn neurons generated from activated NSCs have been identified after SCI [2,5–9].

Myelin associated inhibitors (MAIs), including Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp), largely contribute to creating adverse microenvironments that inhibit axonal regeneration [10]. Studies have shown that MAIs have the ability to induce an intracellular calcium influx, which subsequently enhances epidermal growth factor receptor (EGFR) signaling activation [11,12]. Consequently, EGFR activation is a critical signaling event that contributes to neurite outgrowth inhibition and antagonizing EGFR signaling may promote axon regeneration [11,13]. Our group previously showed

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: zfxiao@genetics.ac.cn (Z. Xiao), zhangsai718@vip.126.com (S. Zhang), jwdai@genetics.ac.cn (J. Dai).
[1] These authors contribute equally to this work.

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that MAIs inhibit neuronal differentiation of neural progenitor cells (NPCs) [14,15]. We discovered that myelin proteins and Nogo-66 (an active fragment of Nogo-A) increase the astrocytic differentiation ratio of NPCs in vitro and that the mTOR-Stat3 pathway is involved in this process [14]. Furthermore, we found that cetuximab cross-linked to collagen sponge increased neuronal differentiation rates of cultured neural progenitor cells (NPCs) when exposed to myelin proteins in vitro [15]. Cetuximab administration markedly induced neuronal differentiation and decreased astrocytic differentiation of transplanted NPCs in rats with acute hemisection SCI [15]. The above studies indicate that the adverse microenvironment within the lesion was the leading cause for neurons only rarely being generated. Therefore, we hypothesized that changing this adverse microenvironment by neutralizing EGFR signaling may induce more neurons within the lesion site, potentially leading to a functional recovery.

Here, we investigated proliferation and migration progresses of endogenous nestin^{+}/BLBP^{+}/Sox2^{+} NSCs in adult rats following complete removal of the thoracic spinal cord at T8 (T8 removal). Because EGFR signaling is largely activated in proliferated NSCs, we verified that blocking EGFR signaling in nestin transgenic mouse with cetuximab, an EGFR signaling antagonist, could promote newborn neuron generation. Because of low antigenicity, excellent biocompatibility and good biodegradability, collagen scaffold is now one of the most popular biomaterial used for acute and chronic spinal cord injury repair research [16–18]. Moreover, the linear ordered collagen scaffold (LOCS) were effectively used as a delivery system in our previous animal SCI repair studies [16–21] and the recent clinical study in human chronic SCI has also successfully proven its safety and feasibility [22]. In this study, a functional scaffold by combining cetuximab, a clinic drug, with linear ordered collagen scaffold was developed and transplanted into the injury site of adult canine 5-mm T8 removal injury model to validate its therapeutic effects on neuronal regeneration and functional recovery. The results showed that implantation of the functional scaffold could effectively promote neurogenesis in the lesion site and enhance motor function restoration in dogs with severe SCI.

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in accordance with Guide for the Care and Use of Laboratory Animals from National Institutes of Health and approved by the Animal Care and Use Committee of the Affiliated Hospital of Logistics University of PAP (AP2014008).

2.2. Animals

Adult female Sprague Dawley rats (200–230 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in temperature and humidity controlled animal quarters under a 12 h light/dark cycle. The Nestin-GFP mice (transgenic mice expressing green fluorescent protein (GFP) under the control of regulatory regions of the nestin gene) were purchased from Cyagen Biosciences Inc (in China). A total of 18 adult female Beagle canines (Anlimo Biotechnology Inc. Jiangsu, China) were normally housed in temperature and humidity controlled animal quarters for at least 7 days.

2.3. NSC culture and differentiation assay

NSCs were cultured as previously described with slight modification [15]. Briefly, the telencephalons or the spinal cord were dissected from newborn or adult SCI rats, respectively. After striping the meninges, tissues were cut into 1 mm^3 pieces, and digested in 0.25% trypsin at 37 °C for 40 min. Then trypsin inhibitor was added to stop the digestion, followed by centrifugation at 500*g* for 5 min. The cell suspension was cultured in 25 cm^2 tissue culture flask (Corning, USA) in serum-free DMEM/F12 medium containing 20 ng/mL bFGF (Peprotech Asia, Rehovot, Israel), 20 ng/mL EGF (Peprotech Asia), 2% B27 (Invitrogen, GIBCO, NY, USA), 30% glucose (Sigma, USA), and 1.83 mg/mL heparin (Sigma, USA). After 7-day culture, neurospheres were enzymatically dissociated to single cells for the following experiments. The NSCs were resuspended in adhesion medium containing 10% FBS and 3×10^5 cells were seeded on 96-well plate pre-coated with 100 g/ml poly-L-lysine. After 1-day culture, the adhesion medium was removed. After wash with PBS three times, differentiation medium containing 2% B27 in DMEM/F12 medium was added.

2.4. The preparation of linear ordered collagen scaffold (LOCS) and functional scaffolds

LOCS was prepared from bovine aponeurosis as described before [23]. Aponeuroses of 0.5 mm thickness were separated from muscles, and then were cut into the proper size. The adjunctive tissues of aponeurosis, including the residual muscles, connective tissues and fats were further removed.

The functional collagen scaffold, 5 mm (3 mm) -long bundle of LOCS fibers were incubated by 50 μL (10 μL) of PBS containing 150 μg (20 μg) cetuximab at room temperature for 30 min. After incubation, the functional collagen scaffolds were implanted into the lesion site of canines (rats).

2.5. Surgery procedures, postoperative care, rehabilitation and grouping of canines

Surgery process was similar as we described before with some modification [24,25]. The canines were anesthetized by subcutaneous administration of atropine sulfate at 0.05 mg/kg and ketamine at 40 mg/kg. The hair on the back of the anesthetized canine was shaved and the skin was cleaned with povidone iodine. Surgery was performed under the sterile conditions. A continuous oxygen supply was supplied to the canine during surgery. Anesthesia was maintained by propofol and physiological saline was used to compensate for blood loss during surgery. A longitudinal incision was made through the skin above the thoracic spine. The back musculature was incised along the midline and dissected away from the vertebral column. A dorsal laminectomy was performed at the level of T7-T9 using bone rongeurs and microscissors. The dura was opened with a surgical blade to expose about 1.5 cm of spinal cord. About 5 mm of T8 spinal cord was removed by performing two complete incisions and removing the intervening tissue. This resulted in about 5 mm long gap in the spinal cord because of retraction. Gelfoam was temporarily placed in the gap to induce hemostasis.

The functional collagen scaffold, 5 mm-long and 5 mm diameter bundle of LOCS fibers alone or binding with 150 μg cetuximab (EGFR antibody), were grafted into the transected spinal cord gap to bridge the defect and the meninge was sutured again after implantation. All animals were allocated into three groups: Control group without implants (n = 6); LOCS group (n = 6) and LOCS + cetuximab group (n = 6).

Dysdefecation and bladder disturbance could be observed immediately after full resection of the spinal cord to a gap of 5-mm long. The bladder had to be emptied manually every 6 h and defecation promoting method was also carried out every 12 h in order to avoid infection. Each canine routinely received antibiotics and glucose intravenously for 5 days and its bladder had to be

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