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A modified collagen scaffold facilitates endogenous neurogenesis for acute spinal cord injury repair

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A B S T R A C T

Due to irreversible neuronal loss and glial scar deposition, spinal cord injury (SCI) ultimately results in permanent neurological dysfunction. Neuronal regeneration of neural stem cells (NSCs) residing in the spinal cord could be an ideal strategy for replenishing the lost neurons and restore function. However, many myelin-associated inhibitors in the SCI microenvironment limit the ability of spinal cord NSCs to regenerate into neurons. Here, a linearly ordered collagen scaffold was used to prevent scar deposition, guide nerve regeneration and carry drugs to neutralize the inhibitory molecules. A collagen-binding EGFR antibody Fab fragment, CBD-Fab, was constructed to neutralize the myelin inhibitory molecules, which was demonstrated to promote neuronal differentiation and neurite outgrowth under myelin *in vitro*. This fragment could also specifically bind to the collagen and undergo sustained release from collagen scaffold. Then, the scaffolds modified with CBD-Fab were transplanted into an acute rat SCI model. The robust neurogenesis of endogenous injury-activated NSCs was observed, and these NSCs could not only differentiate into neurons but further mature into functional neurons to reconnect the injured gap. The results indicated that the modified collagen scaffold could be an ideal candidate for spinal cord regeneration after acute SCI.

Statements of Significance

A linearly ordered collagen scaffold was specifically modified with collagen-binding EGFR antibody, allowed for sustained release of this EGFR neutralizing factor, to block the myelin associated inhibitory molecules and guide spinal cord regeneration along its linear fibers. Dorsal root ganglion neurons and neural stem cells induced by CBD-Fab exhibited enhanced neurite outgrowth and neuronal differentiation rate under myelin *in vitro*. Transplantation of the modified collagen scaffold with moderate EGFR neutralizing proteins showed greatest advantage on endogenous neurogenesis of injury-activated neural stem cells for acute spinal cord injury repair.

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1. Introduction

Spinal cord injury (SCI), followed by permanent sensory loss and volitional movement deficit below the lesion site [1], remains one of the most challenging clinical problems. Central nerve regeneration is limited after injury due to the development of a non-permissive microenvironment [2–7]. Following SCI, a set of complicated pathological processes occur, including myelin-associated inhibitor accumulation, glial scar formation, and cystic cavitation, among others [8,9]. The inhibitory microenvironment is believed to be the predominant underlying cause of the poor neuronal differentiation rate of neural stem cells (NSCs), which contributes to the failure of functional recovery [10,11]. A challenge in effective neurogenesis is how to reconstruct the nerve regeneration microenvironment.

Myelin-associated inhibitors in central nerve injury microenvironments are known to activate the epidermal growth factor receptor (EGFR), subsequently leading to the failure of nerve

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regeneration [12]. Recent studies have focused on blocking EGFR to promote repair after CNS injury [13–15]. Inhibition of EGFR signaling has been shown to reduce the microglial inflammatory response and associated secondary damage after SCI [14]. Our recent report demonstrated that administration of the EGFR neutralizing antibody, cetuximab, improved neuronal differentiation of transplanted NSCs [13]. However, immune rejection of transplanted cells remains an important issue to overcome for therapeutic efficacy. In addition, exogenous cells may influence the effects of targeted therapy on endogenous cells. It is widely believed that NSCs reside in many regions of the adult spinal cord [16–18]. Neuronal loss caused by injury disrupts the ascending and descending spinal tracts, leading to the succeeding dysfunctions of sensory and motor function below the injury site [19,20]. Therefore, in this study, we evaluated the potential of cetuximab to direct neuronal differentiation of endogenous spinal cord NSCs to replenish the lost neurons. However, direct application of cetuximab to the injured site following SCI would likely be subject to rapid diffusion by bodily fluids. A controlled release system should allow storage for sustained drug delivery.

Collagen is a natural protein extracted from animal tissue, which has been characterized as possessing good biocompatibility and excellent biodegradability. Our previous work has shown that several protein factors, when fused with a collagen-binding domain (CBD), could achieve prolonged slow release from collagen [15,21–24]. Therefore, collagen is regarded as an excellent carrier for sustained drug delivery in tissue engineering materials [15,22,25]. In this study, a well-organized collagen scaffold was developed for spinal cord regeneration [26]. In addition to serving as an excellent carrier for drug delivery and as a permissive cell adhesion matrix, the scaffold could also support neurite grow along its ordered fibers [22,26].

Here, we engineered a collagen-binding EGFR antibody (CBD-Fab) by fusing the Fab fragment of cetuximab with a CBD. After characterizing the collagen-binding activity, the blockade to EGFR and the biological activity of CBD-Fab *in vitro* were evaluated, and then the therapeutic effects of CBD-Fab loaded on the collagen scaffold on scar deposition, neuronal differentiation of endogenous NSCs and further maturation were assessed in injured spinal cords by transplantation into severe SCI sites of rats.

2. Experimental methods

2.1. Preparation of CBD-Fab

CBD-Fab was prepared as previously described [21]. The individual *P. pastoris* colonies that integrated the linearized pPICZα-B-CBD-Fab-L and pPIC9 K-Fab-H were analyzed by colony PCR. Ten positive colonies (Mut^+) were confirmed using a small-scale expression trial. The supernatants were collected (12,000 rpm, 30 min) and analyzed via 15% SDS-PAGE. The recombinant CBD-Fab fragment was purified by Ni-NTA affinity chromatography. Under optimized conditions, approximately 2 mg of CBD-Fab antibody fragment with 90% purity was obtained from 1 L of supernatant by nickel affinity chromatography. The Fab without CBD (Natural-Fab, NAT-Fab) was prepared following the same method as CBD-Fab.

2.2. Preparation and characterization of the collagen scaffold

The collagen scaffold was prepared from bovine aponeurosis as previously described [26]. Aponeuroses of 0.5 mm thickness were separated from muscle and cut into the proper size. The adjunctive tissues of the aponeuroses, including the residual muscles, connective tissues, and fats, were removed. The resulting material was denominated as linearly ordered collagen scaffold.

For scanning electron microscopy (SEM), the scaffolds were fixed in 2% glutaraldehyde for 40 min at 4 °C. Subsequently, they were dehydrated in ethanol with the following series of concentrations: 30%, 50%, 70%, 75%, 80%, 90%, and 95% for 10 min, and 100% for 20 min. The ethanol was extracted in 3:1, 1:1, and 1:3 mixtures of ethanol and amyl acetate for 20 min each followed by 100% amyl acetate storage for 20 min. Then, the samples were dried by super critical CO_2 extraction and coated with gold using a sputterer. The microstructure of the scaffolds was observed using a HITACHI S-3000N scanning electron microscope (Hitachi, Japan).

2.3. Collagen-binding assay and sustained release assay of CBD-Fab

The binding ability of CBD-Fab to collagen was measured by a modified enzyme-linked immunosorbent assay (ELISA) according to our previous report [27]. The collagen fibers (4 mm length and 2 mm diameter) were added to a 96-well plate. Subsequently, the plate was blocked with 100 μL of fetal bovine serum (FBS, GIBICO, NY, USA) for 2 h at room temperature. Serial dilutions of NAT-Fab and CBD-Fab (20 μL, 0–10 μM) were added to the wells and incubated at 37 °C for 2 h followed by the removal of unbound proteins with phosphate buffered solution (PBS). An *anti*-histidine polyclonal antibody (1:1000, Sigma Aldrich, Shanghai, China) served as the primary antibody, and a sheep *anti*-mouse-HRP antibody (1:10,000, Sigma Aldrich, Shanghai, China) was used as the secondary antibody. The HRP reaction product was developed following incubation with TMB (Beyotime Institute of Biotechnology, Haimen, China) for 10 min at room temperature. The results were quantified at 405 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). A calibration curve was constructed for each sample to quantitate the total amount of protein retained by the collagen, and the data were analyzed on a Scatchard plot.

The sustained release assay of CBD-Fab from the collagen scaffold was evaluated as previously described [28]. Specifically, collagen fibers (4 mm length and 2 mm diameter) were placed in a 48-well plate, followed by the addition of a 20 μl solution of 5 μg of CBD-Fab or NAT-Fab dissolved in PBS to the dry scaffolds. Following incubation for 30 min at room temperature, the scaffolds were used for the release assay. The scaffolds were soaked in 500 μL of PBS and incubated on a rocker platform at 37 °C and 80 rpm. The PBS in the 48-well plate was replaced every 12 h. Samples were collected at each time point, and the proteins retained on the scaffolds were analyzed by ELISA.

2.4. Myelin preparation

Myelin was extracted from the brains of adult rats using a modified sucrose gradient centrifugation according to a previously published report [29]. Briefly, the brains of four adult Sprague-Dawley (SD) rats were removed and homogenized in 0.32 M sucrose. The suspension was layered over 0.85 M sucrose and centrifuged at 27,000 rpm for 50 min. The crude myelin extraction was collected from the 0.32/0.85 M interface and then placed into chilled sterile water. The suspension and centrifugation procedures were repeated twice in an ice bath. After the removal of excess sucrose, the myelin was re-suspended in chilled sterile water. The resuspended myelin was sterilized by filtration through a 0.22-μm filter (Corning, USA) and stored at −80 °C until use.

2.5. Blockade effect of CBD-Fab to EGFR in vitro

NSCs were isolated and cultured as previously described with a slight modification [13]. Briefly, the telencephalons were dissected from neonatal rats, cut into 1 mm^3 pieces, and digested with 0.25%

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