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VIEWS AND COMMENTARY

The neuronal differentiation microenvironment is essential for spinal cord injury repair

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ABSTRACT. Spinal cord injury (SCI) often leads to substantial disability due to loss of motor function and sensation below the lesion. Neural stem cells (NSCs) are a promising strategy for SCI repair. However, NSCs rarely differentiate into neurons; they mostly differentiate into astrocytes because of the adverse microenvironment present after SCI. We have shown that myelin-associated inhibitors (MAIs) inhibited neuronal differentiation of NSCs. Given that MAIs activate epidermal growth factor receptor (EGFR) signaling, we used a collagen scaffold-tethered anti-EGFR antibody to attenuate the inhibitory effects of MAIs and create a neuronal differentiation microenvironment for SCI repair. The collagen scaffold modified with anti-EGFR antibody prevented the inhibition of NSC neuronal differentiation by myelin. After transplantation into completely transected SCI animals, the scaffold-linked antibodies induced production of nascent neurons from endogenous and transplanted NSCs, which rebuilt the neuronal relay by forming connections with each other or host neurons to transmit electrophysiological signals and promote functional recovery. Thus, a scaffold-based strategy for rebuilding the neuronal differentiation microenvironment could be useful for SCI repair.

KEYWORDS. biomaterials, EGFR, microenvironment, myelin associated inhibitors, neural stem cell, neuronal differentiation, spinal cord injury

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INTRODUCTION

Spinal cord injury (SCI) is a devastating neurologic disorder that often leads to loss of voluntary motor function and sensation below the injury level. SCI initiates a cascade of biochemical reactions that cause continued and pervasive cell death and tissue damage.[1] Inducing neural regeneration and reconstructing neurologic function after SCI remain global challenges. Neural stem cells (NSCs) are a type of multipotent cell that can self-renew and differentiate into neurons and glial cells. NSCs have been identified in and isolated from the brain, and some reports have demonstrated that NSCs also exist in the spinal cord.[2,3] After SCI, quiescent NSCs are activated and migrate toward the injury site.[4-6] Neuronal differentiation of NSCs residing in the spinal cord is regarded as a promising strategy for replenishing lost neurons after SCI. However, endogenous and exogenous transplanted NSCs rarely differentiate into neurons; they primarily differentiate into astrocytes and thus contribute to formation of a glial scar in the injury site, which greatly affects the therapeutic efficacy of NSCs for SCI repair.[7-9] Rebuilding a regenerative microenvironment to promote neuronal differentiation of endogenous or exogenous NSCs is pivotal for the recovery of neurologic function after SCI. Our studies have revealed that a collagen scaffold modified with an anti-epidermal growth factor receptor (EGFR) antibody was able to rebuild a regenerative microenvironment that promoted neuronal differentiation of NSCs; the nascent neurons formed neuronal relays for SCI repair.[6,10-12] Here, we summarize related research to highlight the importance of the neuronal differentiation microenvironment for SCI repair.

The adverse microenvironment present after SCI inhibits neuronal differentiation of NSCs

Although NSCs have been regarded as promising source cells for SCI repair, early clinical trials treating patients with SCI with embryonic stem cells or neural stem cells have not achieved the desired therapeutic effects. For example, the California biotech firm Geron began the first trial of embryonic stem cell therapy for patients with SCI in 2010, but shut down the program in 2011. In 2016, the company Stemcells announced the termination of a phase II clinical study of neural stem cell transplantation for SCI. Many previous animal studies have demonstrated that endogenous and exogenous transplanted NSCs rarely differentiate into neurons, but rather primarily differentiate into astrocytes at the injury site,[13,14] which greatly impairs the therapeutic efficacy of NSCs for SCI repair.

The inhibitive pathophysiological microenvironment present after SCI may be a key factor affecting the therapeutic effects of stem cells in SCI repair. SCI leads to formation of an adverse microenvironment that inhibits axonal regeneration. Myelin-associated inhibitors (MAIs) including Nogo, oligodendrocyte myelin glycoprotein (OMgp) and myelin-associated glycoprotein (MAG) have been identified as the major components of this adverse microenvironment.[15] Moreover, our previous study found that in addition to inhibiting axonal regeneration, MAIs also inhibited neuronal differentiation of NSCs.[16] Nogo-A is a potent myelin-associated protein expressed mainly in oligodendrocytes, which has been identified as an inhibitor of neurite outgrowth in the central nervous system. We discovered that an active fragment of Nogo-A (Nogo-66) expressed on the cell surface inhibited neuronal differentiation of NSCs and promoted differentiation of NSCs into astrocytes. Nogo-66 receptor (NgR) is a glycosyl phosphatidyl inositol (GPI)-linked axonal surface protein that is also expressed in NSCs, which binds Nogo-66 with high affinity to inhibit axon growth. Using NEP1–40, a competitive antagonist of NgR, we demonstrated that NgR activation is involved in the inhibitory effect of Nogo-66 on neuronal differentiation of NSCs.

Small GTPases are critical signaling mediators involved in the inhibition of axonal regeneration by MAIs. MAIs activate the small GTPase RhoA and RhoA-associated kinase (ROCK) to mediate reorganization of cytoskeletal structures, thereby

inhibiting neuronal axon growth. However, the RhoA–ROCK pathway is not involved in the inhibitory effect of Nogo-66 on neuronal differentiation of NSCs, as demonstrated by a study showing that a synthetic inhibitor of ROCK, Y27632, did not prevent Nogo-66-induced inhibition of NSC differentiation. We found that Nogo-66 inhibited neuronal differentiation by activating mammalian target of rapamycin (mTOR) and STAT3[16]. These data provided the first evidence that MAIs inhibit neuronal differentiation of NSCs, and suggest that MAI antagonism might be a potential strategy for promoting neuronal differentiation after SCI.

Collagen scaffolds modified with anti-EGFR antibody prevent inhibition of neuronal differentiation of NSCs by myelin

EGFR is a transmembrane protein expressed in many cells. It is commonly activated by binding of its specific ligands. Upon activation, EGFR dimerization stimulates intracellular protein tyrosine kinase activity, which leads to activation of the MAPK/Akt pathway and thus modulates cell migration, adhesion and proliferation.[17] Previous studies showed that MAIs promoted intracellular calcium influx and activated EGFR signaling.[18,19] Activation of EGFR signaling by myelin is indispensable for inhibition of neurite outgrowth, and attenuating EGFR signaling with the small molecule inhibitor PD168393 improved locomotor and sensory function in a rat model of SCI.[20]

Because EGFR plays a critical role in mediating MAI signaling, we hypothesized that inhibiting EGFR function might prevent inhibition of neuronal differentiation of NSCs by MAIs after SCI. We have investigated the use of biomaterials combined with an anti-EGFR antibody to rebuild an NSC neuronal differentiation microenvironment (Table 1).[6,10-12] A linearly ordered collagen scaffold with excellent biocompatibility and biodegradability, termed NeuroRegen scaffold, was developed to deliver functional biomolecules and stem cells and thus construct a favorable regenerative microenvironment at the site of SCI. The scaffold promoted axonal growth along its collagen fibers and inhibited scar tissue formation.[21] The anti-EGFR antibody interfered with the binding of EGFR to its ligand and thus inhibited EGFR signaling. To avoid rapid diffusion of the anti-EGFR antibody from the collagen scaffold, the antibody was covalently conjugated to the scaffold. Traut's reagent was used to introduce sulfhydryl groups to the collagen scaffold, and then the heterobifunctional crosslinker Sulfo-SMCC was applied to conjugate the amine group of

TABLE 1. Summary of collagen scaffolds modified with anti-EGFR antibodies for spinal cord injury repair.

Scaffolds	Molecules	Cells	Function	Reference
NeuroRegen Scaffold	Chemical conjugated 151IgG, CBD-BDNF	None	Axon regeneration and electrophysiological recovery	Han et al. 2010[11]
Collagen scaffold	Chemical conjuagted Cetuximab	NSCs	Neuronal differentiation, and neuronal relay formation and locomotion function recovery	Li et al. 2013[12]
NeuroRegen Scaffold	CBD-EGFR-Fab	None	Endogenous neuronal differentiation, synaptic formation, myelination, electrophysiological and motor functional recovery	Fan et al. 2017[6]
NeuroRegen Scaffold	CBD-EGFR-Fab	NSCs	NSCs Retainment in SCI sites, neuronal differentiation, synapse formation, electrophysiological and motor functional recovery	Xu et al. 2017[10]

the anti-EGFR antibody to the sulfhydryl group of the collagen scaffold. The collagen scaffold-conjugated anti-EGFR antibody prevented the inhibition of neuronal differentiation of NSCs by myelin.[12]

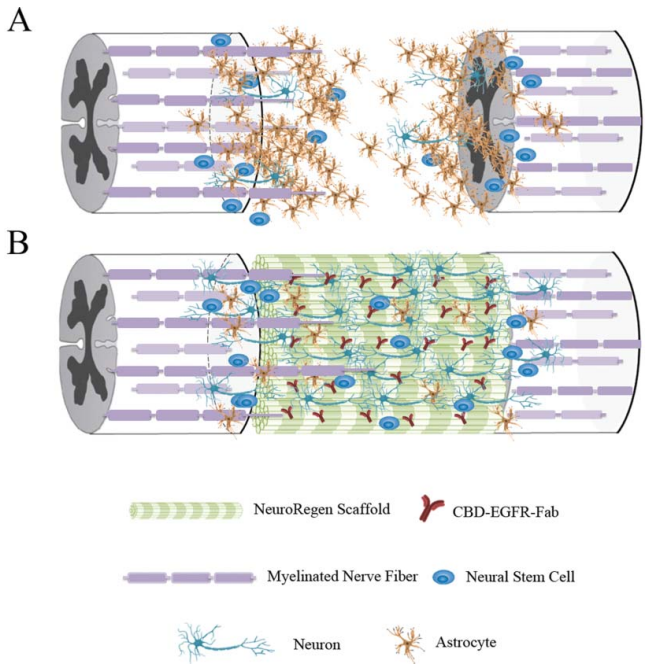
Although chemical conjugation is a convenient method for achieving high levels of anti-EGFR antibody binding to a scaffold, it may affect the biologic activity of EGFR because of the formation of unexpected and excessive intermolecular covalent crosslinking. Our previous studies demonstrated that when multiple recombinant growth factors were tethered to a collagen scaffold via a collagen-binding domain (CBD) introduced by genetic engineering technology, the scaffold exhibited controlled release of the growth factors, and thus promoted repair of different tissue injuries.[22-31] The binding between the CBD and the collagen scaffold imitated the interaction between collagen and collagenase, and the binding force between CBD and collagen was comparable to that between the ligand and receptor.[32] Thus, this approach to achieving controlled release of functional proteins from a collagen scaffold has obvious advantages over chemical crosslinking. We therefore prepared a recombinant CBD–EGFR-Fab protein by fusing the CBD with the fragment antigen-binding (Fab) region of anti-EGFR antibody. The Fab molecule is a 50-kDa fragment of the anti-EGFR antibody that consists of light chain variable and constant domains and a truncated heavy chain, which retains the EGFR-binding ability of its parent antibody but penetrates tissues more quickly because of its lower molecular weight.[33] A functional biomaterial composed of CBD–EGFR-Fab and NeuroRegen scaffold was prepared to promote neuronal differentiation of NSCs. We found that CBD–EGFR-Fab had higher binding affinity for the collagen scaffold than EGFR-Fab without CBD (NAT–EGFR-Fab), and achieved controlled release from the collagen scaffold. CBD–EGFR-Fab inhibited the phosphorylation of EGFR by myelin, which suggests that it blocked myelin-induced EGFR activation in NSCs. The NeuroRegen scaffold combined with CBD–EGFR-Fab prevented the inhibition of neuronal differentiation of cultured NSCs by myelin.[6,10]

Collagen scaffolds modified with anti-EGFR antibody promote neuronal differentiation in animal models of SCI

To evaluate the therapeutic effects of the collagen scaffold modified with anti-EGFR antibody in SCI, we established rat and canine models of complete spinal cord transection. Unlike previous models of SCI that used contusion, compression, dislocation or partial transection, our SCI model had a gap in the spinal cord—the spinal cord was completely separated and no spinal cord tissue was left in the lesion site. This produced a more uniform model that could be used to precisely evaluate the efficacy of biomaterial transplantation.

151IgG is an EGFR-neutralizing antibody that inhibits EGFR signaling activity. 151IgG conjugated to NeuroRegen scaffold using Traut's reagent and Sulfo-SMCC was combined with collagen-binding brain-derived neurotrophic factor (BDNF) (CBD–BDNF) to repair complete spinal cord transection in rats. When transplanted into rats with 6-mm complete spinal cord transection, this scaffold promoted axon regeneration and recovery of electrophysiological function.[11] To explore the neuronal differentiation effects of anti-EGFR antibody in our animal model of SCI, we conjugated a clinical anti-EGFR antibody drug, Cetuximab, to the collagen scaffold. When implanted with exogenous NSCs into rats with 4-mm complete spinal cord transection, this scaffold increased neuronal differentiation and decreased astrocytic differentiation of the transplanted NSCs, and promoted neuronal relay formation and locomotor function recovery. Additionally, we created a canine model of SCI with 5-mm complete transection to examine the therapeutic effects of the NeuroRegen scaffold with Cetuximab. The NeuroRegen scaffold combined with Cetuximab improved locomotion recovery in this canine model of SCI (unpublished), which lays the foundation for future clinical studies.

FIGURE 1. A model of SCI repair. NeuroRegen scaffold modified with CBD–EGFR-Fab promotes neuronal differentiation of neural stem cells (NSCs) for spinal cord injury (SCI) repair. (A) The adverse microenvironment at the site of SCI induces NSCs to differentiate into astrocytes rather than neurons. (B) Transplantation of NeuroRegen scaffold combined with CBD–EGFR-Fab promotes NSC differentiation into mature neurons that form a neuronal relay to bridge the injury gap and thus promote functional recovery in animals with SCI.



In our recent studies, we evaluated the therapeutic outcome of NeuroRegen scaffold combined with CBD–EGFR-Fab in rats with 4-mm complete spinal cord transection. Given that massive activation of endogenous NSCs is observed in SCI sites, we implanted the NeuroRegen scaffold combined with CBD–EGFR-Fab to evaluate its potential for promoting neuronal differentiation of endogenous NSCs. Transplantation of the NeuroRegen scaffold combined with CBD–EGFR-Fab inhibited EGFR phosphorylation in endogenous NSCs in the SCI site and promoted their neuronal differentiation, and ultimately improved synapse formation, myelination, and recovery of electrophysiological and motor function in rats with SCI.[6] The NeuroRegen scaffold combined with CBD–EGFR-Fab also promoted differentiation of exogenous NSCs into neurons in the SCI site. We found that CBD–EGFR-Fab bound to EGFR in NSCs and retained exogenous NSCs at the site of SCI. Moreover, it also promoted differentiation of implanted NSCs into multiple functional neurons and induced synapse formation between grafted and host neurons, which is necessary for recovery of electrophysiological and motor functional recovery after SCI.[10]

CONCLUSIONS AND PERSPECTIVES

SCI repair is challenging because of the inadequate regenerative ability of central nervous system axons and the inhibitive pathophysiological microenvironment present after SCI. The activation and neuronal differentiation of endogenous NSCs is critical for SCI repair. Our studies provide the first evidence that creating a neuronal differentiation microenvironment with biomaterials that attenuate MAI activitypromotes NSC differentiation for SCI repair. The nascent neurons derived from NSCs could form a neuronal relay, which could overcome the bottleneck

problem caused by the inadequate axon regrowth ability of long tracts after SCI. Implantation of the NeuroRegen scaffold could inhibit scar tissue formation and guide the orientated growth of axons along the collagen fibers of the scaffold, which could also help nascent neurons to form a neuronal relay and reconnect with the transected host long tracts at the caudal and rostral segments of the spinal cord.

We propose that functional biomaterials composed of a collagen scaffold and CBD–EGFR-Fab could be used to create a regenerative microenvironment for SCI repair. The specific binding between collagen scaffold and CBD–EGFR-Fab could achieve prolonged and controlled release of CBD–EGFR-Fab. The scaffold may create a comparatively stable microenvironment to help attenuate the inhibitory effects of MAIs on neuronal differentiation. Additionally, a NeuroRegen scaffold composed of longitudinally arranged collagen fibers was used to inhibit scar tissue formation after SCI in our studies. The data demonstrated that the NeuroRegen scaffold inhibited deposition of chondroitin sulfate proteoglycans (CSPGs) in the SCI site. Because CSPGs are one of the main inhibitory molecules expressed by reactive cells after SCI, inhibition of CSPGs by the scaffold provide may allow axon regeneration. This would ultimately contribute to formation of neuronal relays between nascent and host neurons and promote recovery of neurologic function after SCI.

The corticospinal tract (CST) is an important motor nerve of the central nervous system that starts at the cortex and terminates at motor neurons, and controls the movement of the limbs and trunk. It is commonly thought that regeneration of CST fibers is critical for recovery of motor function after SCI. Many efforts have been made to induce long descending tract regeneration, but little evidence has been produced to demonstrate long-distance regeneration of CST fibers crossing the damaged area after SCI. The CST fibers did not regenerate across the lesion in our SCI model in our preliminary work. Thus, instead of CST regeneration, we propose that neuronal relay formation by differentiated functional neurons in the injured long tract axon might be the main mechanism for biomaterial-based SCI repair in our studies. A model of SCI repair was proposed in Fig. 1, endogenous NSCs proliferate and migrate toward the injury site immediately after SCI, but NSCs rarely differentiate into neurons because of the existence of MAIs around the lesion site. After transplantation with a functional scaffold composed of NeuroRegen scaffold and CBD–EGFR-Fab, NSCs differentiated into mature neurons that rebuilt the neuronal relay by forming connections with each other or with host neurons, and thus transmitted electrophysiological signals and promoted functional recovery of animals with SCI.

An in-depth understanding of the spinal cord microenvironment and the neuronal differentiation mechanism of NSCs would allow biomaterial-based strategies for creating a regenerative microenvironment to be optimized. Scaffold-based, precise, spatiotemporally controlled release of functional molecules to promote neuronal differentiation is promising for SCI repair.

ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
CBD	collagen-binding domain
CSPGs	chondroitin sulfate proteoglycans
CST	corticospinal tract
EGFR	Epidermal growth factor receptor
GPI	glycosyl phosphatidyl inositol
MAG	myelin-associated glycoprotein
MAIs	myelin associated inhibitors
mTOR	mammalian target of rapamycin
NgR	Nogo-66 receptor
NSCs	neural stem cells
Omgp	oligodendrocyte myelin glycoprotein
ROCK	RhoA-associated kinase
SCI	spinal cord injury

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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