GM-CSF reduces expression of chondroitin sulfate proteoglycan (CSPG) core proteins in TGF-β-treated primary astrocytes

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GM-CSF plays a role in the nervous system, particularly in cases of injury. A therapeutic effect of GM-CSF has been reported in rat models of various central nervous system injuries. We previously showed that GM-CSF could enhance long-term recovery in a rat spinal cord injury model, inhibiting glial scar formation and increasing the integrity of axonal structure. Here, we investigated molecular mechanisms by which GM-CSF suppressed glial scar formation in an in vitro system using primary astrocytes treated with TGF-β. GM-CSF repressed the expression of chondroitin sulfate proteoglycan (CSPG) core proteins in astrocytes treated with TGF-β. GM-CSF also inhibited the TGF-β-induced Rho-ROCK pathway, which is important in CSPG expression. Finally, the inhibitory effect of GM-CSF was blocked by a JAK inhibitor. These results may provide the basis for GM-CSF’s effects in glial scar inhibition and ultimately for its therapeutic effect on neural cell injuries.

INTRODUCTION

Spinal cord injury (SCI) affects many people and can result in severe neurological damage. Following SCI, the central region of the spinal cord may become critically damaged by primary mechanical injury, developing hemorrhagic necrosis, which expands with time because of the activation of secondary injury processes involving apoptosis (1, 2). Apoptosis plays an important role in neuronal cell death and the loss of neural function immediately after SCI. Thus, preventing apoptosis of neural cells has long been a major therapeutic principle in repairing SCI. Another problem after SCI is factors inhibiting neural regeneration. These include myelin debris-derived proteins, such as Nogo-A and myelin-associated glycoprotein (MAG), and glial scars, composed of reactive astrocytes and a network of chondroitin sulfate proteoglycans (CSPGs). In the case of glial scars, astrocytes expressing glial fibrillary acidic protein (GFAP) become hypertrophic and highly proliferative after SCI and eventually form a dense network of extracellular matrix around the cavity, thereby physically blocking axonal growth and the neural network (3, 4). CSPG core proteins, such as NG2, neurocan, and phosphacan, are also upregulated and become part of the glial scar. Thus, suppression of glial scar formation and CSPG production is believed to provide a favorable environment for axonal regeneration and to be important for the repair of SCI (5).

Granulocyte macrophage colony stimulating factor (GM-CSF) is a well-known hematopoietic cytokine. It was originally identified because of its ability to stimulate the differentiation and function of hematopoietic cells. GM-CSF stimulates bone marrow stem cell proliferation and reduces leukocyte apoptosis while increasing white blood cell numbers in the peripheral blood. Due to its hematopoietic stimulating effects, GM-CSF has been used as a therapeutic cytokine in patients suffering from diseases related to bone marrow suppression. GM-CSF has also been suggested to play important roles in neural systems. GM-CSF induces microglial proliferation and activation, which can induce antigen presentation in neonates and be beneficial for axonal regeneration (6, 7). GM-CSF can also function as a neurotrophic factor and induce the proliferation of neural progenitor cells (NPCs) in vitro (8, 9). The therapeutic effects of GM-CSF are already well known in peripheral nervous system (PNS) injury, where it activates macrophages and Schwann cells to remove myelin debris (10-12). In central nervous system (CNS) injuries, GM-CSF has been shown to activate microglia to remove myelin debris and release brain-derived neurotrophic factor (BDNF), thereby inducing axonal regeneration and functional recovery after brain ischemic injury (13). We have also shown that GM-CSF has neuroprotective and anti-apoptotic activities in rat models of several CNS injuries including SCI, brain ischemia, and Parkinson’s disease (14-19). A study in a rat SCI model particularly showed that GM-CSF not only improved spi-
**RESULTS**

**GM-CSF repressed expression of CSPG core proteins induced by TGF-β3 in primary astrocytes**

TGF-β family proteins are involved in the glial scar formation and have been used to induce the expression of scar genes including CSPG core proteins in an in vitro astrocyte model (21, 22). Primary astrocytes isolated from rats were treated with TGF-β3 for 6 h, and the expression of CSPG core proteins was examined. As shown in Fig. 1, TGF-β3 increased the expression of CSPG core proteins, including NG2, neurocan, and phosphacan, indicating that the astrocyte model of glial scar formation was established. Then, we examined whether GM-CSF could inhibit glial scar formation in the astrocyte model. As shown in Fig. 1A, GM-CSF repressed the TGF-β3-mediated induction of CSPG core proteins in a dose-dependent manner, and GM-CSF receptor antibody abrogated the effects of GM-CSF while G-CSF receptor antibody did not (Fig. 1B). Furthermore, GM-CSF inhibited the TGF-β3-mediated induction of xylosyltransferase (xylT) 1 and 2, which are important in the biosynthesis of CSPG core proteins, but G-CSF had little effect (Fig. 1C). Additionally, G-CSF increased the expression of CSPG core proteins without TGF-β3 treatment, as it did in the TGF-β3-treated astrocytes (Fig. 1B), but GM-CSF did not affect their expression when astrocytes were not treated with TGF-β3 (Fig. 2). Together, these results indicated that GM-CSF can inhibit the TGF-β3-mediated induction of CSPG core proteins through receptor-mediated signal transduction in primary astrocytes, and suggested that GM-CSF may suppress glial scar formation through regulating ex-
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Fig. 3. Effects of GM-CSF and G-CSF on the TGF-β3-induced Rho-ROCK signaling pathway. A, B) Primary astrocytes were treated with TGF-β3 (10 ng/ml) for 24 h with pre-treatment of Rho inhibitor (10 or 25 μM) or ROCK inhibitor (Y-27632: 10 or 25 μM) for 1 h. Then, the expression of neurocan, phosphacan, and NG2 was analyzed. β-actin was used as an internal control. Values below each panel indicate fold normalized expression ratio of each protein to β-actin relative to that of no treatment, taken as 1.

C) Primary astrocytes were treated with TGF-β3 (10 ng/ml) for 24 h with or without pre-treatment of GM-CSF or G-CSF for 6 h, as indicated. Then, the phosphorylation levels of RhoA, ROCK, and MLC were analyzed by Western blotting. Values below each panel indicate fold normalized phosphorylation ratio of each protein to unphosphorylated level of the protein relative to that of no treatment, taken as 1.

DISCUSSION
We demonstrated that GM-CSF could inhibit expression of CSPG core proteins, such as NG2, neurocan, and phosphacan, induced by TGF-β3 in primary astrocytes. Additionally, the expression of CSPG core proteins by TGF-β3 was mediated via activation of the RhoA-ROCK pathway, which was also inhibited by GM-CSF. These results support that the anti-scar forming activity of GM-CSF observed previously in a rat SCI model was mediated by a specific biological action of GM-CSF but not by its neuroprotective effects. Glial scars hinder the exchange of small molecules, cytokines, and neurotrophic factors between lesion areas and adjacent intact neural tissues, which, in turn, inhibits the re-
pair and regeneration of damaged neural tissues (27-29). The scar is composed mainly of various CSPGs secreted by astrocytes, oligodendrocyte precursor cells (OPCs), and meningial cells, and by GFAP-positive astrocytes during the late stage after injury. Many studies have suggested that TGF-β plays an important role in the expression of CSPGs and glial scar formation (22, 30, 31) and a strategy to inhibit TGF-β signaling may be a potential therapy to repair CNS injuries (32). Thus, the specific action of GM-CSF in inhibiting glial scar formation could be a promising therapeutic tool for CNS injuries, including SCI.

The Rho-ROCK signaling pathway is one of the downstream signals of TGF-β (24). Previously, interleukin-1β (IL-1β) was shown to suppress astrogial activation after CNS injury via inhibiting the Rho-ROCK signal pathway (33). Statin, a Rho inhibitor, was shown to inhibit CSPG expression and astrocyte activation, but Y27362, a ROCK inhibitor, increased their expression (34, 35). Thus, the roles of Rho and ROCK in CSPG expression in astrocytes are not yet clearly understood, particularly when cells are treated with TGF-β in vitro. This study first showed that inhibition of Rho and ROCK signals of TGF-β using specific inhibitors or GM-CSF decreased CSPG expression in cultured astrocytes significantly. The exact mechanism(s) of GM-CSF’s effect remain(s) unclear but seem to involve the JAK signal pathway and act on Rho or its upstream pathway of TGF-β signals in astrocytes.

The Rho-ROCK signaling pathway is also implicated in the CSPG inhibition of axon growth and neuronal regeneration (23). Several ROCK inhibitors, such as dimethylfasudil, fasudil, and Y27632, were shown to increase neurite outgrowth in neuronal cells in vitro and axonal regeneration of rat optic nerve in vivo (36, 37). Bacterial C3 exoenzyme, a Rho inhibitor and dominant negative mutant of Rho were also reported to alleviate CSPG-mediated inhibition of neurite outgrowth and to promote neuronal regeneration after injury (38, 39). Having similar inhibitory effects on both Rho and ROCK signals, GM-CSF could inhibit CSPG production in astrocytes and their inhibitory effect on axonal regeneration in neurons.
MATERIALS AND METHODS

Treatment of cytokines and inhibitors
Primary astrocytes were treated with GM-CSF or G-CSF (Chemicon Inc.) for 24 h, alone or in combination with TGF-β3 (Chemicon Inc.). For combinatorial treatments, cells were pre-treated with GM-CSF at 6 h before TGF-β3 treatment. The inhibitors used were LY294002 (PI3K inhibitor; Merck KGaA), statin (Rho inhibitor), Y27632 (ROCK inhibitor; Sigma Chemical Co.), JAK inhibitor I, GM-CSF receptor α antibody, and G-CSF receptor antibody (Santa Cruz Biotech.). The plasmid expressing DN-Ras was kindly provided by Professors Gautam Sethi (National University of Singapore) and Kwang Seok Ahn (Kyung Hee University).

Isolation and culture of primary astrocytes
All animal experiments were performed in the authorized animal care facility and approved by the ‘Committee for the Care and Use of Laboratory Animals’ at Inha University College of Medicine. Primary astrocytes were prepared from cerebral cortices of 1-day old neonatal Sprague Dawley rats. Briefly, dissociated cortical cells were suspended in DMEM (Gibco-BRL) containing 25 mM glucose, 4 mM glutamine, 1 mM sodium pyruvate and 10% FBS, and plated on uncoated T75 flasks at a density of 6 × 10⁵ cells/cm². Monolayer of type 1 astrocytes was obtained 12-14 days after plating. Non-astrocytic cells such as microglia and neurons were detached from the flasks by shaking and removed by changing the medium.

Western blotting
Whole cell lysates were prepared, and Western blotting was performed as described (17). The antibodies were purchased as follows: neurocan and phosphacan from Chemicon Inc., NG2 from Upstate USA Inc., phospho-ROCK from Bios, horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody, RhoA, phospho-RhoA, ROCK, xytT1, xytT2, Raf, and phospho-Raf from Santa Cruz Biotech., MLC, phospho-MLC, Akt, phospho-Akt, STAT5 and phospho-STAT5 from Cell Signaling Technology Inc., and β-actin from Sigma Chemical Co.

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