Neural stem cells and regeneration of injured spinal cord

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Neural stem cells and regeneration of injured spinal cord. Recent progress in the stem cell biology has led much insight into new therapeutic interventions aiming for the regeneration of the damaged central nervous system. The major strategies can be classified into two subgroups: (1) activation of endogenous neural stem cells, and (2) cell transplantation therapies. In either of these strategies, it is crucial to understand the underlying mechanisms of maintenance, activation, and differentiation of neural stem cells and subsequent process, including the migration, survival, and functional maturation of differentiated cells. In this paper, we would like to summarize our recent findings on the therapeutic interventions of the injured spinal cord, especially focusing on the development of treatment for the acute phase of spinal cord injury with anti-interleukin (IL)-6 receptor blocking antibody.

For many years it was thought that once the adult mammalian central nervous system was injured, it never regenerated. However, a paradigm shift is taking place in the light of the progress of the stem cell biology [1, 2]. Previously, our research group has demonstrated the presence of endogenous neural stem-like cells in the adult human brain in collaboration with Pincus et al [3] and Roy et al [4] in the United States. This has given rise to the hope that regenerative capacity persists in the adult mammalian central nervous system, and, in fact, neurogenesis has been shown to be induced when the central nervous system is exposed to damage due to cerebral ischemia, etc. As a result, activation of endogenous stem cells is now regarded as another important central nervous system regeneration pathway in addition to cell transplantation.

INJURED SPINAL CORD AND NEURAL STEM CELLS

Nevertheless, despite the presence of endogenous neural stem cells in the adult mammalian spinal cord, neurogenesis is not thought to occur there, not only under intact conditions, but even when injured [5]. Moreover, it is also suggested that when the spinal cord is damaged, undifferentiated cells that are positive for the intermediate filament nestin and derived from cells close to the central canal in the vicinity of the lesioned site vigorously proliferate, migrate around the lesioned site, and differentiate into astroglia. In other words, although endogenous neural stem cells are present in the spinal cord and they do proliferate after a spinal cord injury, almost all of them differentiate into astroglia, not into neurons or oligodendroglia, which are myelin-forming cells. Furthermore, since the astroglia form glial scars around cysts as time passes after the injury, regeneration of neuronal axons disrupted by the injury, extension of axons past the injury site, and remyelination of demyelinated neural axons seemed to be impossible.

Nevertheless, since neural stem/progenitor cells derived from the adult mammalian spinal cord were shown to cause neurogenesis when transplanted into the hippocampus, which is a neurogenic site [6]. Thus, the phenomenon of the adult mammalian spinal cord being nonneurogenic appears to be attributable to the microenvironment in the spinal cord of adult mammals and not to be a cell-autonomous phenomenon of spinal-cord–derived neural stem/progenitor cells. A study of changes in the microenvironment in the injured spinal cord after spinal cord injury in rats revealed that expression of the mRNAs of a variety of proinflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-1β, and IL-6] increased 6 to 12 hours after the injury and peaked by 4 days [7]. These proinflammatory cytokines were already known to exhibit cytotoxicity, including induction of apoptosis of neurons and oligodendrocytes; however, we focused our attention on the induction of IL-6 expression, because leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF), which belong to the IL-6 cytokine superfamily, are known to induce neural stem/progenitor cells to differentiate into astroglia by activating the gp130/Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway [8]. Because of that, the IL-6 whose expression is induced in the spinal cord in the acute phase of the injury was expected to act on endogenous neural stem cells and induce them to differentiate into (reactive) astroglia. The reactive astroglia induced in the injured spinal cord are known to express chondroitin sulfate proteoglycans (CSPGs),
which are axonal growth inhibitors, and to inhibit axonal regeneration [9].

**BLOCKADE OF IL-6 SIGNALING IN EXPERIMENTAL SPINAL CORD INJURY**

Based on the above mentioned grounds, it was expected that inhibiting signal transduction by the IL-6 induced in the spinal cord in the acute phase of the injury would inhibit the production of reactive astrogliosis and of axonal regeneration inhibitors, such as CSPGs, and would be linked to induction of regeneration of the central nervous system. Administration of chondroitinase ABC, which breaks down these CSPGs, to rats with spinal cord injury has, in fact, been reported to be effective in regenerating axons and restoring function after injuries [9], and the aspects of IL-6 as a proinflammatory cytokine cannot be overlooked either. The concept of so-called “secondary injury,” a self-destructive tissue injury mechanism associated with spinal cord injury in which the blood-spinal cord barrier breaks down, inflammatory cells such as macrophages and lymphocytes infiltrate the spinal cord, and the infiltration is followed by secondary scar formation, cavitation, and demyelination has long been advanced. High-dose synthetic adrenocortical steroid therapy (methylprednisolone) in the acute phase is currently performed as the sole method of preventing this secondary spinal cord injury, but its safety and efficacy have been questioned. Because IL-6 plays a major role in the differentiation of a variety of cells, including B lymphocytes, T lymphocytes, and monocytes, it was hoped that inhibition of signal transduction by IL-6 would have an inhibitory effect on secondary spinal cord injury, and it was against this background that we investigated whether there was any behavioral improvement by blocking the IL-6 signal with anti-IL-6R blocking antibody.

Next, we investigated the effect of the IL-6 signal on gli scar formation in vivo in an actual spinal cord injury in mice [10]. First, a laminectomy was performed at the T9 level, and a spinal cord injury model was created in the form of a contusion injury model by allowing a 3 g weight with a 1.2 mm diameter tip to drop from a height of 25 mm. Anti-IL-6R blocking antibody was then injected intraperitoneally in one shot (a similar dose of rat IgG was injected in the control group), and we (1) investigated expression of molecules related to the IL-6 signal by the immunoblot method 12 hours after the injury, (2) 2 weeks later, immunostained tissue sections for various markers, investigated the degree of reactive astrogliosis and macrophage infiltration, and investigated whether neurogenesis had been induced, and (3) investigated whether there was any behavioral improvement by 6 weeks after the injury (Fig. 1).

**Investigation of the IL-6 signal**

To confirm the presence of so-called “secondary injury,” a self-destructive tissue injury mechanism associated with spinal cord injury in which the blood-spinal cord barrier breaks down, inflammatory cells such as macrophages and lymphocytes infiltrate the spinal cord, and the infiltration is followed by secondary scar formation, cavitation, and demyelination has long been advanced. High-dose synthetic adrenocortical steroid therapy (methylprednisolone) in the acute phase is currently performed as the sole method of preventing this secondary spinal cord injury, but its safety and efficacy have been questioned. Because IL-6 plays a major role in the differentiation of a variety of cells, including B lymphocytes, T lymphocytes, and monocytes, it was hoped that inhibition of signal transduction by IL-6 would have an inhibitory effect on secondary spinal cord injury, and it was against this background that we investigated whether there was any behavioral improvement by blocking the IL-6 signal with anti-IL-6R blocking antibody.

To confirm that the IL-6 signal had been blocked in the injured spinal cord, 12 hours after the injury we used the immunoblot method to check for phosphorylation of STAT3, which is a molecule downstream from the IL-6 signal, and since it was also found to be significantly reduced in the anti-IL-6R blocking antibody group, we were able to confirm that the preparation that was intraperitoneally injected in one shot immediately after the injury had acted in the injured spinal cord.

**Histological examination**

To quantitatively evaluate reactive astrogliosis, 5-bromo-2′-deoxyuridine (BrdU) was intraperitoneally injected daily for 2 weeks after the injury, and the reactive gliosis was quantitatively evaluated in the form of BrdU/GFAP double positive cells. The results showed that reactive astrogliosis in the center and periphery of the lesion had been inhibited in the anti-IL-6R blocking antibody group. On the other hand, a recent series of studies has demonstrated that differentiation of neuron or astroglia from neural stem cells is regulated in a mutually exclusive mechanism [8, 12]. More specifically, it is now understood that a complex (CBP/p300-phosphorylated Smad complex) in which a transplantation factor that acts downstream of the bone morphogenetic protein (BMP) signal [i.e., active Smad (phosphorylated Smad)], and the transplantation coactivator CBP/p300 are bound in neural stem/progenitor cells, which are capable of differentiating into both the neuronal lineage and the astroglia lineage, and that when the CBP/p300-phosphorylated Smad complex forms a functional complex with phosphorylated STAT3, they differentiate into astroglia lineage cells, whereas when it forms complexes with a pronuclear-type
Experimental protocol:

IL-6R antibody group
Control group
MR16-1 (rat IgG) 100 µg/g ip immediately after SCI
purified rat IgG  same amount as above

Administration: Single shot just after the injury by i.p. injection

- Reduction of reactive astrocytes and microglial invasion in the lesion sites.

Fig. 1. Administration of anti-interleukin (IL)-6 receptor antibody (MR16-1) into spinal cord injured animals. Immediately after the contusion spinal cord injury was induced at the T9 level, mice were intraperitoneally injected with a single dose of MR16-1 or with the same volume and concentration of purified rat IgG (control group). To quantify the IL-6–mediated signaling, at 12 hours after the injury, the amount of phosphorylated signal transducers and activators of transcription (STAT3) within the spinal cord tissue of the lesion epicenter was measured by immunoblotting. At 2 weeks after the injury, animals in both the MR16-1 group and the control group were immunohistochemically characterized. By the 6 weeks after the injury, three different tests were used to assess recovery of motor function after the injury [10].

bHLH factor, such as neurogenin1/2, they differentiate into the neuronal lineage. Since we observed a decrease in phosphorylated STAT3 in the anti-IL-6R blocking antibody group, we hypothesized that “if neurogenin-expressing cells are present in injured spinal cord, it is also possible that CBP/p300-phosphorylated Smad complex will have a chance to bind to neurogenin1/2 in the anti-IL-6R blocking antibody group, and for neurogenesis to be induced.” We expect that under certain conditions neurogenesis also occurs in the spinal cord of adult mammals, and that it is not an “absolute nonneurogenic site,” as had previously been thought. The experimental demonstration of neurogenesis in the adult central nervous system is regarded as a finding that indicates just how important the microenvironment is. We are currently investigating this possibility.

In addition, to assess the inhibitory effect of anti-IL-6R blocking antibody administration on the inflammatory response, 2 weeks after the injury, we quantitated the degree of inflammatory cell infiltration by immunostaining spinal cord tissue sections for Mac1 (CD11b), a marker of inflammatory cells, including macrophages [10]. The results showed that infiltration by Mac1-positive cells in the anti-IL-6R blocking antibody group had been inhibited to one third the level of infiltration in the control group. We quantitatively determined the size of the connective tissue scars, which are thought to occur as a result of inflammatory cell infiltration, and compared them between the anti-IL-6R blocking antibody group and control group, but the region was significantly smaller at the center of the lesioned site in anti-IL-6R blocking antibody group. Based on these results, it appeared that blocking the IL-6 signal had inhibited the inflammatory reaction and secondary injury, and that a greater amount of normal tissue had spared from destructive injury.

Behavioral analysis

Since the model of spinal cord injury was a model of spinal cord contusion injury at the T9 level, we observed hind limb paralysis. We therefore assessed efficacy in the anti-IL-6R blocking antibody group by three different evaluations of motor function: spontaneous hind limb motor function (BBB score), vertical righting, and the Rota-Rod treadmill test to assess recovery of forelimb-hind limb function. The results showed significantly more favorable recovery in the anti-IL-6R blocking antibody group than in the control group, and it was especially noteworthy that the anti-IL-6R blocking antibody that had been administered in one shot immediately after the injury contributed to long-term motor improvement (observations until 6 weeks after the injury, which is the chronic phase in the mouse). This is important because it means that appropriate management and treatment in the acute phase can have a major impact on the long-term outcome. The mechanism of the phenomenon of improvement in the motor function of the hind limbs in response to administration of the antibody is thought to have been that, by inhibiting the formation of glial scars, it may have inhibited expression of axonal growth inhibitors contained in them and promoted axonal regeneration as a result, and that a great deal of normal tissue was spared by the mitigation of secondary injury.

IL-6 has been thought to have a protective action on nerve cells in vitro. There have even been reports of the size of brain ischemia being reduced by administration of
IL-6 [13], and its action seems to have varied considerably according to the pathology, degree of expression of the IL-6 signal, and the timing. However, when the IL-6 signal was overexpressed after spinal cord injury, its action as a proinflammatory cytokine greatly exceeded its neuroprotective effect, and the result was an increase in the extent of the injury and severe impairment of axonal regeneration instead [14]. The possibility that IL-6 exerts a neurotrophic action from the subacute phase to the chronic phase after the injury cannot be ruled out. However, because of its short half-life of only several days, the anti-IL-6R blocking antibody has been postulated to block the acute-phase neurotoxic IL-6 signal alone, without interfering with the subsequent neuroprotective action. Thus, we believe that it displays a very logical pharmacologic action. In other words, through multiple mechanisms, including inhibition of astrogliosis by endogenous neural stem cells, induction of neurogenesis, and inhibition of mobilization of inflammatory cells to the injured spinal cord, depending on the varied functions that IL-6 possesses, anti-IL-6R blocking antibody shows promise of providing a new type of regeneration therapy in the acute phase of spinal cord injury. Humanized monoclonal antibody against human IL-6R (MRA) (Atlizumab) possesses an excellent IL-6 signal-inhibiting effect. Its safety and metabolic distribution and tolerance are being investigated in detail, and clinical research in the treatment of inflammatory diseases, such as rheumatoid arthritis, Crohn’s disease, Castleman’s disease, has progressed [15–17]. It is currently regarded as capable of becoming an acute-phase treatment for spinal cord injury instead of high-dose steroids, whose efficacy has been questioned, and we are setting up a system that will enable early clinical studies.

THERAPEUTIC TIME WINDOW FOR STEM CELL TRANSPLANTATION INTO INJURED SPINAL CORD

The findings that proper regulation of IL-6 signaling is beneficial for treatment of spinal cord injury are also relevant to determine the therapeutic time window of the neural stem/precursor cell transplantation into the injured spinal cord [1, 2]. Previously, we showed that adult spinal cords are not absolutely nonpermissive for neurogenesis from the transplanted neural stem/precursor cells, however, and that a narrow therapeutic time window can allow successful transplantation [18]. This brief window of opportunity might arise because the microenvironment in the host spinal cord changes rapidly after the injury. As already mentioned, during the acute phase, which immediately follows the injury, the levels of many pro-inflammatory cytokines (such as IL-1, IL-6, and TNF-α) that have neurotropic or astrocyte-inducing effects, increase and then decline sharply within 24 hours [7], indicating that the microenvironment of the acute phase is not suitable for survival and/or neuronal differentiation of grafted cells [1, 2]. However, the microenvironments appear to change into a more favorable situation for neuronal differentiation and survival afterward. In fact, the transplantation of in vitro expanded neural stem/precursor cells results in mitogenic neurogenesis when the transplantation into the injured adult rat spinal cord is carried out 9 days after injury, but not when the transplantation is done within a few days of the injury [18]. The chronic phase of spinal cord injury is not likely to be appropriate for therapeutic transplantation due to the lack of inducing factor for neurogenesis [Okada H, et al, submitted for publication] or due to formation of enlarged cysts and the development of glial scarring, which might inhibit axonal regeneration [1, 2].

Taken together, we can conclude that the microenvironments within the injured spinal cord are major determinants for the fates of both endogenous neural stem cells and exogenously transplanted neural stem/precursor cells. These facts would give us deep insights for development of new therapeutic interventions.

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