

Chapter 2

Microenvironment Within the Injured Spinal Cord Focusing on IL-6

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Abstract In recent years, a variety of studies have been conducted towards the goal of achieving regeneration of the central nervous system using neural stem cells. However, various complex factors are involved in the regulation of neural stem cell differentiation, and many unresolved questions remain. It has been reported that after spinal cord injury, the intrinsic neural stem cells do not differentiate into neurons but into astrocytes, resulting in the formation of glial scars. Based on reports that the expression of IL-6 and the IL-6 receptor is sharply increased in the acute stages after spinal cord injury and that IL-6 may serve as a factor strongly inducing the differentiation of neural stem cells into astrocytes, we examined the effects of an antibody to the IL-6 receptor in cases of spinal cord injury and found that the antibody indeed suppressed secondary injury (caused by inflammatory reactions) and glial scar formation, facilitating functional recovery. In this paper, we present the data from this investigation and discuss the relationship between IL-6 signals and spinal cord injury.

Keywords Glial scar • IL-6 • Regeneration • Spinal cord injury

2.1 Introduction

The annual incidence of traumatic spinal cord injury (SCI) in Japan is about 40 per 1,000,000 population. Every year, about 5,000 individuals sustain SCI in this country. Recent advances in patient management during the acute stages of SCI have dramatically reduced the death rate from SCI. However, the total number of patients

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with SCI suffering from complications, such as permanent paralysis of the extremities, sensory disturbances, bladder/bowel disturbances, and bedsores, is reported to be more than 100,000 in Japan. The treatments currently administered to patients with spinal cord injuries are not designed to cure paralysis but for control of systemic factors during the acute stages of injury, stabilization of dislocations and fractures by surgical decompression, reduction or fixation, and prevention of worsening of paralysis; none of these treatments affect the spinal cord itself. Of course, the number of patients who are able to resume social activities through rehabilitation beginning soon after injury has been increasing. On the other hand, there are many patients who are forced to be discharged severely paralyzed from the hospital. In the chronic stage, patient management is focused on rehabilitation, and no active treatment is provided other than expectant therapy for complications. In the 1990s, the effectiveness of massive doses of steroids in the acute stages of SCI was reported based on the results of animal studies and mass clinical studies. This therapy, however, was subsequently proven to be less effective than was initially reported. In recent years, several reports have been published describing adverse reactions to this therapy or casting doubts on the effectiveness of this therapy. At present, more than 10 years after it was first introduced, the need is felt for review of this steroid therapy [1].

Over the past decade, significant progress has been made in the field of stem cell biology pertaining to the central nervous system, and it has been revealed that neural stem cells are seen not only during the intrauterine period but also during adulthood. Furthermore, it is now possible to isolate and incubate these stem cells [2]. Neural stem cells are cells with self-copying potential and the capability of differentiation into diverse cell groups (neurons, astrocytes, oligodendrocytes, etc.). The mammalian central nervous system is an outcome of asymmetrical division of neural stem cells and sophisticated and complex interactions among these cells (involving secretory factors) during embryonic development. It has long been believed that the central nervous system can never regenerate after injury. However, there is now growing concern about regenerative medicine (tissue engineering) techniques aimed at inducing regeneration of the degenerated or injured central nervous system and restoration of its functions by reproducing the central nervous system generation process using intrinsic or extrinsic neural stem cells [3]. In this paper, we shall present our data and discuss the effects of IL-6 signals on the inflammatory reactions and intrinsic neural stem cells in the presence of SCI.

2.2 Neural Stem Cells and IL-6 Signals in Spinal Cord Injury

It has been shown that neural stem cells are also present in the spinal cord of mature mammals, but that in the event of injury, these neural stem cells do not differentiate into neurons but into astrocytes instead, to form glial scars [4]. Glial scars are primarily composed of activated astrocytes and express large amounts of chondroitin sulfate proteoglycan (CSPG) that suppresses the growth of axons. These scars are thus considered to be a great physical and chemical obstacle against axonal regeneration.

In a study using rat models with SCI, it was demonstrated that treatment with chondroitinase ABC, which can degrade CSPG, was useful in promoting axonal regeneration and functional recovery following SCI [5].

Based on the contention that changes in the microenvironment within the injured spinal cord could play an important role in the differentiation of neural stem cells exclusively into astrocytes, we examined the time-course of changes in the mRNA expression levels of various cytokines during the acute stages of SCI in rats, using RNase protection assays. This analysis revealed that while the expression of TGF- β (an anti-inflammatory cytokine) showed a subacute increase, reaching its peak 4–7 days after the injury, the expression of inflammatory cytokines (IL-1 β , IL-6 and TNF- α) showed acute increase, reaching a peak within 12 h after the injury [6]. In particular, the SCI group exhibited an approximately 30-fold increase of IL-6 expression as compared to the sham-operated group. Regarding the signal transduction related to IL-6, it is known that while the expression of IL-6 activity is very weak in the presence of IL-6 alone, the complex formed by the binding of IL-6 to the IL-6 receptor serving as a ligand binds to gp130 (a membrane-bound receptor), leading to signal transduction to cells (trans-signaling) [7]. Because of this unique form of signal transduction, an increase in the expression of the IL-6 receptor is a key factor determining signal transduction related to IL-6. We quantified the expression of the IL-6 receptor by Western blotting in C57/B6 mice with SCI caused by compression at the level of the ninth thoracic vertebra and found that there was an approximately eightfold increase in the expression of this receptor within 12 h after the injury as compared to the level before the injury [8]. We paid close attention to this sharp increase of IL-6 and IL-6 receptor expression in the acute phase of SCI, based on the contention that this might be one of the factors responsible for the differentiation of neural stem cells into astrocytes and not neurons after SCI.

Neural stem cells are induced by interactions among various factors. In this connection, it has been found *in vitro* that IL-6 signals, including LIF and CNTF, act on neural stem cells to powerfully induce their differentiation into astrocytes [9]. This finding has also been endorsed in studies *in vivo*. In one such study, IL-6-knockout mice showed suppression of astrogliosis following SCI [10]; in another, mice showing excessive expression of IL-6 and the IL-6 receptor showed marked gliosis even after mild injury of the spinal cord [11]. It has also been shown that injury of the spinal cord resulted in a marked decrease of axonal growth in mice with excessive IL-6 signals as compared to that in intact mice [12]. On the basis of these previous findings, we contended that suppression of IL-6 signals in the acute stage of SCI might suppress the formation of glial scars. We, therefore, conducted a study in mice using a monoclonal antibody directed against the mouse IL-6 receptor (MR16-1), jointly with Chugai Pharmaceutical Co. Ltd [8].

First, we examined the effects of IL-6 signals on the differentiation of neural stem cells intrinsically present in the spinal cord. To this end, we collected neural stem cells from the spinal cords of 8-week-old mature mice and incubated them *in vitro* for 3 days to induce differentiation. In the control group, the cells showed scarcely any growth of cellular processes. In the group treated with IL-6 and the IL-6 receptor, however, marked growth of astroglial processes was noted. The percentage of

cells differentiating into GFAP-positive astrocytes was also higher in the IL-6 + IL-6 receptor treatment group. This result can be interpreted as indicating that IL-6 signaling does indeed significantly stimulate the differentiation of neural stem cells into astrocytes, as reported previously. However, when the cells were incubated in the presence of both IL-6 and MR16-1 (an antibody directed against the IL-6 receptor), the effect of the IL-6 signals was attenuated. These results suggest that the blocking of the IL-6 signals with antibody directed against the IL-6 receptor can suppress the differentiation of intrinsic neural stem cells into astrocytes *in vivo*.

Then, we examined the effects of IL-6 signals on the formation of glial scars *in vivo*, using a mouse model of SCI. First, the spinal cord of the mouse was exposed at the level of the ninth thoracic vertebra; then, a 3-g weight was dropped from a height of 25 mm on to the exposed dura matter to induce contusion SCI. The mouse was given a single intraperitoneal injection of the IL-6 receptor antibody immediately after SCI. Two weeks later, specimens of spinal cord tissue were immunostained with various markers. In the mice with SCI, no softening or void formation was seen in the tissue specimens, unlike in rats and other models. Instead, large scars replacing the gray matter were found at the center of the injured spinal cord. In a previous study, this scar was characterized as being composed of connective tissue rich in type IV collagen and fibronectin [13]. When immunostained, the scar was found to contain no neurons, but groups of inflammatory cells. GFAP-positive glial scars were formed surrounding these scars composed of connective tissue. To mark the newly formed cells after SCI, we administered an intraperitoneal injection of bromodeoxyuridine (Brd-U), which is a substrate for DNA synthesis, to the animals for 14 consecutive days after the induction of SCI, and quantified the glial scars by using astrocyte formation as an indicator by double-staining with Brd-U and GFAP. This study revealed that the glial scar formation was suppressed at the center of the injured spinal cord. The number of double (Brd-U/GFAP)-positive cells was 25 % lower in the group treated with the IL-6 receptor antibody immediately after the injury than in the control group treated with IgG alone. To confirm that the IL-6 signals had actually been blocked, we examined the phosphorylation (activation) of STAT3 (a transcription factor acting in the IL-6/IL-6 receptor/gp130 signal pathway) by Western blotting and found a significant decrease of STAT3 phosphorylation in the IL-6 receptor antibody treatment group. This result endorses the proposition that the drug administered as a single intraperitoneal injection immediately after SCI acts on the injured spinal cord.

2.3 Inflammatory Cytokines in Spinal Cord Injury and Their Relationship to Secondary Injury

In addition to its effects on the neural stem cells, we shall also discuss the role of IL-6 as an inflammatory cytokine in cases of SCI. In regard to the pathogenesis of SCI, the concept of secondary injury was proposed many years ago. This concept proposes that self-destroying tissue damage occurs secondary to primary

mechanical damage caused by the external force in cases of SCI. Histopathological examination has demonstrated that small bleeding spots due to enhanced vascular permeability are formed within 10 min after SCI and that edema, ischemia, and focal bleeding develop in the gray and white matter within a few hours after injury, causing progressive necrosis of the surrounding nerve cells due to the shortage of nutrients and oxygen. Clinically, paralysis associated with SCI is the most severe immediately after the injury. In cases where paresis develops following SCI, gradual recovery from paresis is seen for some period after the very acute phase. It is thus unknown to what precise extent secondary injury is involved in the paralysis seen after SCI. However, many reports of animal studies have demonstrated that suppression of secondary injury, for example, by the use of steroids, resulted in some alleviation of the paralysis. Therefore, reduction of secondary tissue damage is a major goal of treatment in cases of SCI [14, 15]. Factors known to stimulate the aforementioned self-destructive responses include NO, free radicals, glutamic acid, and MMP. In particular, the inflammatory cytokines seem to be closely involved in the secondary damage. Of course, increased or suppressed expression of a variety of cytokines has been demonstrated in the presence of SCI, and it is known that these cytokines have two distinctive effects (neurotoxic and neurotrophic effects). Although we need to be careful while interpreting the effects of these cytokines in cases of SCI, previous studies have suggested that the marked increase of IL-6 expression during the ultra-acute stage of SCI plays a central role in the inflammatory reactions and serves as a neurotoxic factor.

Therefore, to examine the effects of an antibody to the IL-6 receptor on the severity of these inflammatory reactions, we stained spinal cord tissue specimens (collected 2 weeks after injury) with Mac1 (CD11b), a marker of inflammatory cells, and quantified the degree of inflammatory cell infiltration. This analysis showed that the infiltration by Mac1-positive cells was decreased to 1/3 in the IL-6 receptor antibody treatment group as compared to that in the control group. This result endorses the reported finding that forced expression of IL-6 signals after SCI resulted in an approximately sixfold increase of neutrophil infiltration and twofold increase of macrophage infiltration [12]. Furthermore, the recent study demonstrated that temporal blockade of IL-6 signaling after SCI abrogates damaging inflammatory activity and promotes functional recovery by promoting the formation of alternatively activated M2 macrophages [16, 17]. In mice with SCI, connective tissue scars are formed, in contrast to the softening and void formation observed in the spinal cord tissue of rats and other animals. It has been shown that the size of such connective tissue scars is related to the severity of the SCI [13]. In our study also, the size of the connective tissue scars was quantified and compared between the control group and the IL-6 receptor antibody treatment group; the comparison revealed a significantly decreased size of the scars at the center of the injured spinal cord in the antibody-treated group. These results suggest that blocking of the IL-6 signals can suppress inflammatory reactions and secondary injury, thus attenuating injury of intact tissue.

On the basis of these findings, we examined the effects of IL-6 receptor antibody treatment on the functional recovery after SCI. The IL-6 receptor antibody treatment group showed significantly better recovery than the control group in terms of

three different motor functions (spontaneous leg exercise, vertical standing, and continued exercise on a rotating rod). The possible reasons for this difference are: (1) suppression of glial scar formation suppressed the expression of axonal growth inhibitory factors present in the glial scars leading to stimulation of axonal regeneration, and (2) reduction of secondary damage allowed more intact cells to be protected from injury. Another possible mechanism is that suppression of the differentiation of neural stem cells into astrocytes leads to a relative increase in the efficiency of differentiation of these cells into neurons.

2.4 For Clinical Application

The relationship between IL-6 and SCI has been discussed above. IL-6 is a cytokine with diverse physiological activities. It is extensively involved in various phenomena occurring *in vivo*, including inflammation, immunity, and regulation of cell differentiation [7]. IL-6 was first identified as a factor involved in the differentiation of B cells into antibody-producing cells. Later, it was revealed that IL-6 is also involved in the differentiation and proliferation of not only B cells, T cells, and monocytes but also fibroblasts, osteoclasts, hematopoietic stem cells, and neural stem cells. It has also been shown that excessive IL-6 expression is involved in many diseases, such as rheumatoid arthritis, Crohn's disease, and Castleman's disease. A human-type monoclonal antibody to the IL-6 receptor has been developed jointly by Osaka University and Chugai Pharmaceutical Co. Ltd., for use in the treatment of rheumatoid arthritis. This antibody has exhibited excellent therapeutic efficacy in clinical trials [18]. This human-type monoclonal antibody (MRA, Atlizumab) has a potent inhibitory activity against IL-6 signals. Its safety profile and pharmacokinetic characteristics, such as its metabolism, distribution, and tolerance, have been evaluated in depth and already introduced clinically. This antibody is thus very close to the stage of clinical application for SCI.

The half-life of this human-type antibody *in vivo* is relatively short (about 7 days). This feature is ideal when considering the expression of IL-6 in cases of SCI. As illustrated above, IL-6 exerts both neurotrophic and neurotoxic effects, and it has been shown to protect nerve cells *in vitro* [19]. Considering the report that IL-6 treatment reduced the size of the cerebral infarct in cases of brain ischemia [20], it seems likely that IL-6 exerts variable activities depending on the condition, degree of IL-6 signal expression, and the timing of its administration. When excessive IL-6 was expressed after SCI, the activity of this cytokine as an inflammatory cytokine was much greater than its neurotrophic activity, leading to an increase in the area of injury and marked suppression of axonal regeneration [11]. This indicates that excessive IL-6 signals are neurotoxic, at least in the acute stages of SCI. However, we cannot rule out the possibility that it serves as a neurotrophic (neuroprotective) factor in the subacute to chronic stages of SCI. The IL-6 receptor antibody with a short half-life *in vivo* may be expected to block only the neurotoxic IL-6 signals in the acute stages

of SCI and not to inhibit the neuroprotective signals during the subsequent phases of SCI. Therefore, this antibody offers promise as a means for suppressing the expression of neurotoxic IL-6 signals alone in cases of SCI.

2.5 Conclusions

Although various methods of treating central nervous system injury have been studied, no method for radical treatment of central nervous system injury has yet been established. Neural stem cells are receiving close attention as a tool for reconstruction of the injured neuronal network. However, there are many unresolved questions concerning the mechanism of regulation of their differentiation and functional recovery in cases of SCI. These issues need to be studied in further detail from various viewpoints. The IL-6 receptor antibody has been shown to modify the microenvironment around the injured spinal cord by alleviating the post-injury tissue destruction or secondary damage and glial scar formation. To achieve reconstruction of the injured neural network, the most practical method may be the combined use of such an environment-modifying factor and cell transplantation [3, 21–23]. We believe that an accumulation of basic studies in this field would allow regeneration and reconstruction of the spinal cord after injury in the near future.

Conflict of Interest The author declares that he has no conflict of interest.

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