Interleukin-6 induces proliferation in adult spinal cord-derived neural progenitors *via* the JAK2/STAT3 pathway with EGF-induced MAPK phosphorylation

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Abstract. *Introduction:* In a previous study, we observed cell proliferation 3 days after spinal cord injury, and levels of interleukin-6 (IL-6) and epidermal growth factor (EGF) had significantly increased in the region of the injury. *Objectives:* The purpose of the new study described here was to evaluate the roles of IL-6 and EGF after traumatic damage to the spinal cord having isolated neural progenitor cells (NPC) from adult mice. *Methods and results:* Evidence provided by the trypan blue dye exclusion assay, 5-bromodeoxyuridine immunoreactivity and Western blot analysis indicated that IL-6 and EGF induced proliferation of these spinal cord-derived NPCs via phosphorylation of Janus-activated kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) and mitogen-activated protein kinases (MAPK), respectively. Combined treatment with IL-6 and EGF accelerated proliferation of cells synergistically and phosphorylation of STAT3 and extracellular signal-regulated kinase 1/2 (Erk1/2). Furthermore, AG490 and AG1478, JAK2 inhibitor and EGFR inhibitor, respectively, prevented the IL-6- and EGF-induced proliferation of the cells. Interestingly, IL-6-activated MAPKs but EGF did not influence JAK2/STAT3 activation; AG490 specifically inhibited IL-6-induced Erk1/2 phosphorylation without affecting IL-6-induced phosphorylation of Raf and MEK1/2. These results indicate that IL-6 is directly involved in Erk1/2 activation via JAK2 and that Erk1/2 provides a signal bridge between the IL-6-induced JAK2/STAT3 pathway and EGF-induced MAPK pathway. *Conclusions:* Our study is the first demonstration of IL-6- and EGF-stimulated proliferation of spinal cord progenitor cells via JAK2/STAT3 and MAPK signalling pathways. These pathways play key roles in repopulation and regeneration of spinal cord tissue after injury. It may represent novel therapeutic targets for pharmacological intervention in central nervous system disease, including spinal cord injury.

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INTRODUCTION

Traumatic spinal cord injury (SCI) causes permanent neurological deficit due to loss of spinal cord neurons and their axons. It has been demonstrated that the neurological damage is not due to an intrinsic inability of neurons to regenerate, but rather to the unfavourable environment (David & Aguayo 1981). There are three phases to SCI response that occur after the injury: the acute phase, a phase of secondary tissue loss and the chronic phase (Hulsebosch 2002). The initial injury destroys many of the local neurons and glia; in addition, it triggers secondary tissue damage in a few days through production of free radicals, excessive release of excitatory neurotransmitters, and apoptotic cell death (Smith *et al.* 1998; Sakurai *et al.* 2003; Crack & Taylor 2005). All of these contribute to conduction malfunction (Taoka & Okajima 1998). Despite the original cell loss, cell proliferation increases in a few days after SCI (Zai *et al.* 2005), and cell density returns to near normal levels by 6 weeks later (Rosenberg *et al.* 2005). This repopulation may be due in part to proliferation of local neural and glial progenitors that divide in response to the injury (Horner *et al.* 2000; McTigue *et al.* 2001). Some studies have reported that multipotent neural progenitor/stem cells are present in the adult spinal cord and they can be isolated (Quinn *et al.* 1999; Liu *et al.* 2006). They are known to respond to epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) (Mansergh *et al.* 2000), and constitutively, they produce various cytokines such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor- α (TNF- α) (Klassen *et al.* 2003). It has been suggested that these cytokines contribute to the spontaneous recovery after SCI by stimulating proliferation of local progenitor cells that help repopulate the injured cord (Zai & Wrathall 2005).

Interestingly, IL-6 rapidly increases after SCI (Hayashi *et al.* 2000). In addition, IL-6 induced the functional recovery of neurons by activation of the JAK/STAT pathway (Yamauchi *et al.* 2006). On the other hand, IL-6 has been shown to be involved in neuronal death and differentiation (Bonni *et al.* 1997; Gadiant & Otten 1997). Thus, it is difficult to link cell repopulation and functional recovery by IL-6 after SCI. IL-6 acts on target cells by binding to its receptors (IL-6R), which form complexes with gp130 (Taga & Kishimoto 1997). Such binding induces activation of Janus-activated kinases (JAK), leading to phosphorylation of docking sites of the receptor for SHP2 (SH2 domain-containing tyrosine phosphatase) and signal transducers and activators of transcription (STAT) (Heinrich *et al.* 1998). STAT3 is one of these and it plays crucial roles in various intracellular signalling cascades involved in cell proliferation and differentiation (Ihle 2001). Phosphorylated STAT3 molecules form dimers and are subsequently translocated to the nucleus, where they lead to transactivation of their target genes (Schindler & Darnell 1995). Furthermore, IL-6 has been known to activate mitogen-activated protein kinases (MAPK) (Ishikawa *et al.* 2006). Because of the diverse physiological responses of IL-6, elucidation of signalling mechanisms involved in the IL-6-induced regulation of cell population growth needs to be clarified. In addition, several reports have demonstrated that interferon- γ (IFN- γ), and tumour necrosis factor- α (TNF- α) as well as EGF trigger JAK/STAT pathways in normal and cancer cells (Darnell *et al.* 1994; Guo *et al.* 1998; Gallmeier *et al.* 2005). However, very little is known about functional JAK/STAT pathways in spinal cord derived NPCs and injured spinal cord tissue.

In our previous study, we found that cells at the epicenter of the injury region proliferated significantly after SCI and that transcription factors of the JAK/STAT family were strongly expressed in that region. In addition, IL-6 and EGF were significantly elevated in a similar pattern of cell proliferation there (Ahn *et al.* 2006). This was represented as an early attempt at spinal cord repair and regeneration under the influence of IL-6 and/or EGF. Thus, in this study,

to investigate potential correlation between cell proliferation and IL-6 and/or EGF expression after SCI, we have isolated spinal cord-derived NPCs from adult mouse spinal cord and evaluated the effect of IL-6 and/or EGF on the proliferation of these cells. We investigated expression of JAK and STAT proteins and their regulation in response to IL-6 and/or EGF. Our study is the first demonstration that IL-6 and/or EGF stimulates proliferation of spinal cord-derived NPCs and that the distinct signalling pathways including JAK2/STAT3 and MAPK play key roles in cytokine-induced proliferation of mouse spinal cord NPCs after traumatic injury.

MATERIALS AND METHODS

Animals and materials

CD-1[®] (ICR) mice 4 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS) and foetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA); recombinant human IL-6 and EGF were obtained from Sigma (St. Louis, MO, USA); anti-phospho-JAK2, anti-phospho-STAT3, anti-phospho-Raf, anti-phospho-MEK1/2 and anti-phospho-Erk1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA); anti-actin, anti-Nestin and anti-Tuj antibodies were provided from Sigma; anti-glial fibrillary acidic protein (GFAP), anti-A2B5 and anti-5-bromodeoxyuridine (BrdUrd) were provided from DAKO (Hamburg, Germany), Chemicon (Hampshire, UK) and Sigma, respectively; the JAK2 and EGF receptor inhibitors, AG490 and AG1478, respectively, were from Calbiochem (San Diego, CA, USA); trypan blue, BrdUrd, dimethyl sulfoxide and other reagents were also purchased from Sigma.

Culture and characterization of spinal cord-derived NPCs

Spinal cord-derived NPCs were isolated from 4-week-old adult ICR mice, according to the protocol previously described by Weiss *et al.* (1996). To isolate the cells, mouse spinal cord tissue was placed in HBSS at 4 °C without Ca²⁺ or Mg²⁺ and was washed extensively with equal volumes of HBSS. Tissue pieces were minced into small pieces (~1 mm³) and enzyme digested at 37 °C for 20 min with 0.25% trypsin. Enzyme activity was neutralized with DMEM, containing 10% FBS and tissue was centrifuged at 1200 *g* for 10 min, to obtain a high cell density in the pellet. Cultured NPCs were then incubated overnight at 37 °C in 5% CO₂ in 10% FBS containing DMEM. Media were replaced after the first 48 h and every fourth day thereafter. After passage 2 or 3, cells were used for characterization of their differentiation capacity. To verify the neurogenic characteristics of NPCs, they were subjected to differentiation conditions. Cultured undifferentiated NPCs formed spherical clumps and free-floating neurospheres. Spheres of the cells were transferred to a Petri dish and were cultured in neurobasal (NB) medium (Invitrogen, Gaithersburg, MD, USA) and supplemented with B27 (Invitrogen), 20 ng/mL basic FGF, and 20 ng/mL EGF (Sigma) for 4–7 days. Culture density of the spheroid bodies was maintained at 10–20 cells/cm² to prevent self-aggregation. For neural differentiation, the neurospheres were layered on PDL-laminin double-coated chamber slides (Lab-Tek, Nalge/Nunc International, Rochester, NY, USA). Spheres were cultured and maintained for 10 days in NB media containing 2% FBS, 10 µM retinoic acid, and B27 supplement. During differentiation, 70% of the media was replaced every 4 days. All data collected were representative of at least three different experiments. For identification of differentiated NPCs, we performed real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and immunocytochemistry using neural marker primers and primary antibodies.

Expression of target gene transcripts

Before and after neural differentiation, total cell RNA was isolated with trizol (Life Technologies, Frederick, MA, USA) and was reverse transcribed into first-strand cDNA using oligo-dT primer, amplified by 35 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min) of PCR using 20 pM of specific primers. PCR amplification was performed using the primer sets. All primer sequences were determined using established human GeneBank sequences for genes indicative of neural lineages or control genes. For analysis of neural marker expression in NPC control cells, we also performed real time RT-PCR following same procedure. For labelling of PCR product, we used Syber green detection kit purchased from Applied Biosystems (Foster, CA, USA). Quantitative RT-PCR was performed using an ABI7700 Prism Sequence Detection System. Primers sequences were designed using Primer Express software (PE Applied Biosystems, Warrington, UK) using gene sequences obtained from the GeneBank database.

Gene	Forward sequence	Reverse sequence
β -actin	5'-TCCTTCCTGGGTATGGAATC-3'	5'-ACTCATCGTACTCTGCTTG-3'
Nestin	5'-ACCAAGAGACATTTCAGACTCC-3'	5'-CCTCATCCTTATTTTCCACTCC-3'
GFAP	5'-GCTCGATCAACTCACCGCCAACAA-3'	5'-GGGCAGCAGCGTCTGTCAGGTC-3'
Myelin basic protein	5'-ACACGGGCATCCTTGACTCCATCGG-3'	5'-TCCGGAACCAGGTGTTTTCAGCG-3'

Quantifying cell proliferation

Monolayer spinal cord-derived NPCs were harvested with trypsin, re-suspended at 5×10^5 cells/mL, and seeded on to 24-well plates in DMEM supplemented with 10% FBS. After reaching semiconfluence, the cells were washed with HBSS. Medium was then replaced with serum-free medium, and incubation was continued overnight. Serum-starved NPCs were treated with various concentrations of IL-6 (1–40 ng/mL) and EGF (1–40 ng/mL) for 6 days, and counted using a haemocytometer (Fauconneau *et al.* 2002). In addition, to determine effects of AG490 or AG1478 on cytokine-induced proliferation, serum-starved NPCs were pre-treated with AG490 or AG1478 (10 μ M, respectively) for 30 min, followed by treatment with EGF (20 ng/mL) or IL-6 (20 ng/mL) for 6 days; total numbers of cells were counted. Mean values for each treatment group were obtained from four to six samples obtained in three independent experiments.

Immunocytochemical detection of spinal cord NPCs and measurement of BrdUrd incorporation

In order to determine proliferation capacity of spinal cord-derived NPCs after treatment with cytokines, cells were seeded at a density of 2×10^5 cells/well in 12-well plate, in standard proliferation medium, either alone or supplemented with EGF (20 ng/mL) or IL-6 (20 ng/mL). After 24 h, BrdUrd (10 nM) was added to the cultures for 24 h. At the end of the culture period, cells were fixed with 4% paraformaldehyde solution for 30 min and were permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After extensive washing with phosphate-buffered saline (PBS), cells were blocked at room temperature for 30 min with 1% normal goat serum. Cells were then incubated overnight at 4 °C in blocking solution containing primary antibodies: anti-GFAP (1 : 1500), anti-Nestin (1 : 200), anti-Tuj (1 : 250), anti-A2B5 (1 : 200) and anti-BrdUrd (1 : 250). After extensive washing in PBS, the cells were incubated for 30 min with FITC, Texas red, or TRITC-conjugated secondary antibodies (1 : 250, Molecular Probe, Eugene, OR, USA). Results were then analysed using a fluorescence microscope (Leica Microsystem, Exton, PA, USA). The percentage of NPCs with BrdUrd incorporated into their nuclei was calculated as replicating cells to total number of cells per field. Percentages represent the means of at least three separate determinations, in each of which a minimum of 1000 cells was counted.

Whole tissue extract preparation and Western blot analysis

For Western blotting, we prepared eight groups: treated (i) without IL-6 or EGF, (ii) with 20 ng/mL IL-6 only, for 1 h, (iii) with 20 ng/mL EGF only, for 1 h, (iv) with 20 ng/mL IL-6 and 20 ng/mL EGF for 1 h, (v) with 20 ng/mL IL-6 for 1 h after pre-treatment with 10 nM AG490 for 30 min, (vi) with 20 ng/mL EGF for 1 h after pre-treatment with 10 nM AG1478 for 30 min, (vii) with 20 ng/mL IL-6 for 1 h after pre-treatment with 10 nM AG1478 for 30 min, and (viii) with 20 ng/mL EGF for 1 h after pre-treatment with 10 nM AG490 for 30 min. Briefly, 5×10^5 cells were washed twice in cold DPBS and lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM ethyleneglycoltetraacetic acid, 1 mM glycerophosphate, 1 mM Na_3VO_4 , and 1 mM phenylmethylsulphonyl fluoride). Lysates were clarified by centrifugation at 15 000 *g* for 10 min and total protein content was determined using the Bio-Rad (Milan, Italy) protein assay kit. For Western blotting, equal amounts (40 μg) of protein extract in lysis buffer were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and were transferred to nitrocellulose membranes. Membranes were pre-incubated with PBS containing 5% skimmed milk, and were probed with anti-GFAP (1 : 2000), anti-Nestin (1 : 1000), anti-Tuj (1 : 1000), anti-p-Raf (1 : 1000), anti-p-MEK1/2 (1 : 1000), anti-p-Erk1/2 (1 : 1000), anti-p-STAT3 (1 : 1000), anti-p-JAK2 (1 : 1000) and anti- β -actin (1 : 2000) in PBS containing 5% skimmed milk, overnight at 4 °C. Following incubation with primary antibodies, membranes were washed with PBS and were subsequently incubated for 1 h with IgG horseradish peroxidase-conjugated secondary antibodies (1 : 1000). Signals were detected using the ECL chemiluminescence detection kit (Amersham, Freiburg, Germany). Relative band intensities were determined using Quality-one-4, 2, 0 image analyzer (Bio-Rad, Hercules, CA, USA) and were normalized to the actin immunoblot.

Statistical analysis

All data were expressed as means $\pm$ standard deviation (SD) from five or more independent experiments. Statistical analysis was performed by two-tailed Student's *t*-test for independent variables. Significance was determined with $P < 0.05$.

RESULTS

IL-6- and EGF-stimulated proliferation of primary mouse spinal cord-derived NPCs

To investigate a potential correlation between cell proliferation and IL-6 and EGF expression after injury induction, we performed a dose-response study of the effects of recombinant human form of IL-6 and EGF on these progenitor cells. The cells proliferated similarly in response to IL-6 and EGF, and the stimulatory effect of EGF on proliferation was more potent than that of IL-6. Maximal proliferation was induced at 20 ng/mL cytokines and a high concentration (>20 ng/mL) of cytokine led to reduction of proliferation of the NPCs (data not shown). Treatment of the cells with 20 ng/mL cytokine increased the cell number in a time-dependent manner. After 6 days, cell numbers were increased by IL-6 and EGF by about 3.5-fold and 4.2-fold, respectively, compared to untreated controls. It was observed that IL-6 and EGF could act directly on the spinal cord cells (Fig. 1) and IL-6- and EGF-induced proliferation of NPCs.

To further evaluate the proliferation states of the cells by IL-6 and EGF, we examined the distinctive spatio-temporal patterns of BrdUrd incorporation. The cells were treated with IL-6 (20 ng/mL) and EGF (20 ng/mL) for 1 day then were fixed and stained for BrdUrd, Nestin,

Figure 1. Effect of IL-6 family on proliferation of spinal cord-derived NPCs. NPCs derived from spinal cords were treated with IL-6 (20 ng/mL) (a) or EGF (20 ng/mL) (b) for 6 days. Cell proliferation was determined by trypan blue dye exclusion assay, as described in the Materials and Methods section. Values are expressed as percentage of control, which was defined as 100%. Mean values for each treatment group were obtained from four to six samples obtained in three independent experiments. Statistical analysis was performed by a two-tailed Student's *t*-test for independent variables. Data are plotted as the mean $\pm$ SD (**P* < 0.05 versus control).

GFAP, A2B5, or Tuj. The number of cells with each phenotype was compared between BrdUrd⁺ cells and BrdUrd⁻ cells in response to exogenous cytokines. The results showed that BrdUrd immunoreactivity was undetectable in controls but dramatically increased in the cytokine-treated groups. Cultured NPC-derived neurospheres consisted of A2B5-positive oligodendrocyte progenitor cells, GFAP-positive glial progenitor cells, Tuj-positive neurones and Nestin-positive precursor cells based on phenotypic markers (supplementary Figure 1). As shown Fig. 2, percentages of BrdUrd⁺ and Nestin⁺ populations in the cultures significantly were increased by IL-6 and EGF treatment. In addition, these cytokines were shown to be strong mitogens for glial progenitors (GFAP⁺ cells) and their levels were perhaps partially responsible for observed increase in density of BrdUrd⁺ precursor cells.

STAT3 and Erk1/2 are involved in cytokine-induced proliferation of spinal cord-derived NPCs

It has been previously documented that JAK/STAT and MAPK pathways induced by IL-6 and EGF, respectively, play a crucial role in cell proliferation (Kamimura *et al.* 2003). To gain insight into the implication of such cytokine-induced proliferation, we evaluated effects of JAK2 or EGFR inhibitors on proliferation of the cells. In addition, to observe IL-6-induced or EGF-induced signal pathways, we determined phosphorylation of STAT3 or Erk1/2, reflecting JAK/STAT or MAPK pathways, respectively, by Western blotting.

Figure 2. Immunocytochemical analysis of BrdUrd-incorporated cells in cultured. Spinal cord-derived NPCs were cultured for 24 h with IL-6 (20 ng/mL) (a) or EGF (20 ng/mL) (b) in the presence of 10% FBS, pulsed with BrdUrd for 4 h, and the percentage of cells incorporating BrdUrd were determined for each culture as described in the Materials and Methods section. BrdUrd-positive nuclei in five sections were randomly chosen and showed an average percentage of positive cells. Statistical analysis was performed by a two-tailed Student's *t*-test for independent variables. Data are plotted as the mean $\pm$ SD (**P* < 0.05 versus control).

Serum-starved NPCs were exposed to IL-6, and the phosphorylation level of STAT3 was determined by Western blotting. STAT3 was phosphorylated in response to treatment with IL-6, and maximal phosphorylation of STAT3 occurred at 30 min of NPC exposure to IL-6 (Fig. 3a). In addition, phosphorylation of STAT3 was attenuated by pre-treatment of cells with AG490, the JAK2-specific inhibitor (Fig. 3b). Furthermore, the IL-6-induced proliferation of the cells was completely abolished by pre-treatment with AG490 (Fig. 3c). Similar results were obtained using EGF (Fig. 4). When NPCs were treated with EGF, Erk1/2 was maximally phosphorylated at 30 min by EGF (Fig. 4a). EGF-induced phosphorylation of Erk1/2 in NPCs was completely prevented by pre-treatment with AG1478, the EGFR inhibitor (Fig. 4b); in addition, AG1478 treatment decreased the cells' proliferation (Fig. 4c). This indicated that activation of both STAT3 and Erk1/2 is necessary for the proliferation of spinal cord NPCs.

Figure 3. Role of STAT3 in the IL-6-induced proliferation of spinal cord NPCs. (a) Serum-starved NPCs were exposed to 20 ng/mL IL-6 for the indicated time. Phosphorylation and expression levels of STAT3 in cell lysates (30 μg) were determined by Western blotting with anti-phospho-STAT3. (b) Serum-starved spinal cord NPCs were pre-treated with various dose of AG490 for 30 min. (c) Proliferation was probed by trypan blue dye exclusion assay after 3 days. Data are expressed as percentage of control (vehicle-treated cells), which was defined as 100%. Statistical analysis was performed by a two-tailed Student's *t*-test for independent variables. Data are plotted as the mean ± SD (**P* < 0.05 versus control; #*P* < 0.05 versus IL-6).

To investigate the involvement of IL-6 and EGF on proliferation, the NPCs were grown in a combination culture medium containing IL-6 and EGF for 6 days. Combination of IL-6 and EGF increased the cells' proliferation more than IL-6 or EGF alone (Fig. 5a). In addition, phosphorylation levels of Erk1/2 and STAT3 were increased by co-treatment with IL-6 and EGF, but STAT3 phosphorylation was not affected by EGF (Fig. 5b). On the other hand, in order to evaluate phenotype expression of the cells that had proliferated by IL-6 or EGF action, we determined the protein expressions of Nestin, GFAP and Tuj by Western blotting. As shown in Fig. 5c, expressions of Nestin and GFAP were increased by IL-6 or EGF, whereas expression of Tuj by the two types of cytokine was not increased. Treatment by IL-6 or EGF increased protein expression of Nestin by 34% or 81%, while GFAP protein increased by 13% or 21%, respectively, only. These findings clearly indicate that NPCs derived from spinal cords can be propagated extensively by treatment with IL-6 or EGF. Similarly, phosphorylational changes after combination treatment with IL-6 and EGF (Fig. 5b), protein expression of Nestin and GFAP increased by co-treatment with the cytokines. Relative to controls, Nestin and GFAP significantly increased 107% and 32%, respectively. These results further indicate that IL-6 and EGF stimulate proliferation of these spinal cord NPCs through the activation of STAT3 and Erk1/2.

Figure 4. Role of Erk1/2 in the EGF-induced proliferation of NPCs. (a) Serum-starved spinal cord NPCs were exposed to 20 ng/mL EGF for the indicated time. The phosphorylation and expression levels of Erk1/2 in cell lysates (30 μ g) were determined by Western blotting with anti-phospho-Erk1/2. (b) Serum-starved spinal cord NPCs were pre-treated with various dose of AG1478 for 30 min. (c) Proliferation was probed by trypan blue dye exclusion assay. Data are expressed as percentage of control (vehicle-treated cells), which was defined as 100%. Statistical analysis was performed by a two-tailed Student's *t*-test for independent variables. Data are plotted as the mean $\pm$ SD (**P* < 0.05 versus control; #*P* < 0.05 versus EGF- β).

Erk1/2 provides a signal bridge between the IL-6-induced JAK2/STAT3 pathway and the EGF-induced MAPK pathway

To further investigate the roles of STAT3 and Erk1/2 activation on IL-6- or EGF-induced proliferation, serum-starved spinal cord-derived NPCs were pre-treated with AG490 or AG1478. Next, we observed activation of JAK/STAT and MAPK signal pathways by Western blotting analysis (Fig. 6). As shown in Fig. 6a, IL-6-activated Erk1/2, as well as JAK2/STAT3 and AG490, specifically inhibited IL-6-induced Erk1/2 phosphorylation without affecting IL-6-induced phosphorylation of Raf and MEK1/2. However, in the presence of AG1478, EGF did not alter levels of active JAK2 and STAT3, although Raf, MEK and Erk1/2 were significantly down-regulated (Fig. 6b). These results indicate that IL-6 is directly involved in Erk1/2 activation, which represents a downstream target for the EGF-induced MAPK pathway. To confirm the specific cross-talk between IL-6 and EGF signalling, we investigated the effect of AG1478 on IL-6-induced activation of the MAPK pathway as well as the JAK/STAT pathway (Fig. 7). Although IL-6 has been shown to activate both STAT3 and Erk1/2, EGF did not influence STAT3 activation in NPCs. Interestingly, EGF-induced MAPK activation did not decrease in the presence of the JAK2 inhibitor, AG 490. Treatment with AG490 resulted in inhibition of EGF-induced phosphorylation of Erk1/2. These results indicate that IL-6 induces Erk1/2 activation *via* JAK2, and that Erk1/2 provides a signal bridge between the IL-6-induced JAK2/STAT3 pathway and the EGF-induced MAPK pathway.

Figure 5. Synergy proliferation effects of IL-6 and EGF in cultured NPCs. (a) Cells were treated with IL-6, EGF or IL-6 together with EGF. After 3 days, cell proliferation was probed by trypan blue dye exclusion assay as described in the Materials and Methods section. Data are expressed as percentage of control (vehicle-treated cells), which was defined as 100%. (b) The activation of STAT3 and Erk1/2 in NPCs stimulated with bFGF, IL-6 or bFGF together with IL-6 for 30 min was detected as Western blotting analysis using the indicated antibodies. (c) Serum-starved NPCs were pre-treated with IL-6, EGF or IL-6 together with EGF for 6 days and the phenotype protein expression was detected with Western blotting analysis using the indicated antibodies. Expression of proteins was quantified by using the Quality-one-4, 2, 0 image analyzer as described in the Materials and Methods section. Values are expressed as percentage of control, which was defined as 100%. Statistical analysis was performed by a two-tailed Student's *t*-test for independent variables. Data are plotted as the mean $\pm$ SD (* P < 0.05 versus control).

Figure 6 Effects of IL-6 and EGF on JAK/STAT and MAPKs signal pathways in NPCs. (a) Serum-starved spinal cord NPCs were pre-treated with AG490 for 30 min was stimulated with IL-6 for 30 min, and then followed by Western blotting with indicated antibodies. (b) Serum-starved spinal cord NPCs were pre-treated with AG1478 for 30 min was stimulated with EGF for 30 min, respectively, and then followed by Western blotting with indicated antibodies. Expression of proteins was quantified by using the Quantity-one-4, 2, 0 image analyzer as described in the Materials and Methods section. Values are expressed as percentage of control, which was defined as 100%. Statistical analysis was performed by a two-tailed Student's *t*-test for independent variables. Data are plotted as the mean $\pm$ SD (* P < 0.05 versus control; # P < 0.05 versus IL-6 or EGF).

DISCUSSION

After SCI, approximately 50% of cells at the site of injury die within 1 day (Grossman *et al.* 2001). However, cell proliferation is up-regulated by day 3 (Zai *et al.* 2005), and returns to near normal levels by 6 weeks after injury (Rosenberg *et al.* 2005). This repopulation of cell density may be due to activity of local endogenous NPCs after traumatic injury of spinal cord (Horner *et al.* 2000; McTigue *et al.* 2001; Zai & Wrathall 2005). Recently, Yamauchi *et al.* (2006) reported that IL-6 produced in the injured spinal cord at the acute stage, induced recovery of

Figure 7. Effects of EGFR inhibitor and JAK2 inhibitor on IL-6-induced signal pathway in NPCs. Serum-deprived NPCs were pre-incubated with 10 μ M of the EGFR inhibitor AG1478 or JAK inhibitor AG490 for 30 min before the addition of IL-6 and EGF. The phosphorylation and expression levels of protein were determined by Western blotting with indicated antibodies. Expression of proteins was quantified by using the Quality-one-4, 2, 0 image analyzer as described in the Materials and Methods section. Values are expressed as percentage of control, which was defined as 100%. Statistical analysis was performed by a two-tailed Student's *t*-test for independent variables. Data are plotted as the mean $\pm$ SD (**P* < 0.05 versus control; #*P* < 0.05 versus IL-6 or EGF).

motor function after SCI. Interestingly, it has been reported that NPCs predominantly differentiate into astrocytes and oligodendrocytes instead of the appropriated neurons lost after SCI (Yamamoto *et al.* 2001; Takahashi *et al.* 2003). Several reports have demonstrated that IL-6 served as a factor strongly inducing differentiation of NPCs into astrocytes (Klein *et al.* 1997; Nakamura *et al.* 2005). Therefore, it was unclear whether IL-6 would be directly involved in repopulation of injured spinal cord. Furthermore, it may not provide the responsible mechanisms of recovery in IL-6-induced cell proliferation. In this study, NPCs derived from the spinal cord of adult mouse were actively caused to proliferate by IL-6 as well as EGF (Fig. 1). In addition, IL-6 or EGF induced proliferation of Nestin⁺ and GFAP⁺ progenitor cells (Fig. 2). It was suggested that IL-6 or EGF might contribute to the spontaneous recovery observed after traumatic injury of spinal cord by stimulating proliferation of local progenitor cells (Wennerstein *et al.* 2004; Zai & Wrathall 2005).

It is well documented that IL-6 and EGF induce JAK/STAT3 and MAPK pathways in NPCs, respectively (Reynolds & Weiss 1992; Kitchens *et al.* 1994). Although constitutive activation of MAPKs by EGF contributed to the NPCs' proliferation (Kojima & Tator 2000, 2002), IL-6-regulated NPCs proliferated either positively or negatively, according to the alternation in the

microenvironment (Nakamura *et al.* 2005). Particularly, IL-6-induced STAT3 activation plays a crucial role in various intracellular signalling cascades involved in cell proliferation and differentiation (Schindler & Darnell 1995; Bonni *et al.* 1997; Gadiant & Otten 1997; Ihle 2001). In this study, IL-6- or EGF-induced proliferation of spinal cord-derived NPCs *via* JAK/STAT or MAPK pathways, respectively. Furthermore, AG490 or AG1478 prevented the IL-6- or EGF-induced proliferation of NPCs, respectively (Figs 3 and 4). These data suggest that IL-6 or EGF is associated with anti-apoptotic signals and survival in mouse spinal cord-derived NPCs after SCI. STAT3 activation is mediated by various JAKs (Schindler & Darnell 1995), especially cytokine-induced JAK2 activation and subsequently phosphorylated STAT1 and STAT3 (Kamimura *et al.* 2003). Several reports have indicated that JAK2/STAT1 activation mediates cell death induced by cytokines (Meydan *et al.* 1996; Gorina *et al.* 2005), whereas JAK2/STAT3 activation is associated with cell proliferation (Hattori *et al.* 2001). It has been indicated that fine equilibria occur between JAK2 activation and the different proteins of the STAT family, and underlies specificity of regulation of gene transcription and of their subsequent biological effects (Gorina *et al.* 2005). In the present study, we observed that IL-6 elicited activation of JAK2 and STAT3 in NPCs. Furthermore, pharmacological blockage of JAK2 activation, AG 490, completely prevented IL-6-induced phosphorylation of STAT3 and cell proliferation in these cells (Figs 3 and 6). These results suggest that IL-6 may play an equivalent role in regulation of proliferation of NPCs through a JAK2/STAT3-dependent pathway. Further studies using IL-6 gene knockout mice may allow us to be able to decipher the role of the JAK2/STAT3 signalling pathway in response to SCI.

Fukada *et al.* (1996) have demonstrated that activation of both the JAK/STAT and MAPK pathways is necessary for mouse pro-B-cell proliferation. IL-6 activated the MAPK pathway through phosphorylation of the SH2 domain of STAT (Heinrich *et al.* 1998). Therefore, inhibition of the MAPK cascade or JAK/STAT pathway resulted in growth inhibition (Grandis *et al.* 2000). However, in other studies, the MAPK pathway is also involved in negative regulation of signal transduction through the JAK/STAT pathway (Symes *et al.* 1997; Kim *et al.* 1998; Schaper *et al.* 1998). As shown in Fig. 5 of our study, co-treatment with IL-6 and EGF accelerated cell proliferation and phenotype expression of NPCs. In addition, IL-6 activated Erk1/2 activation, which represents a downstream target for the EGF-induced MAPK pathway, as well as Erk1/2 activation was prevented by AG490, a specific JAK2 inhibitor (Fig. 6). These results indicate that IL-6 is directly involved in Erk1/2 activation *via* JAK2, and that there is cross-talk between the IL-6-induced JAK2/STAT3 pathway and the EGF-induced MAPK pathway (Sriuranpong *et al.* 2003; Ishikawa *et al.* 2006). Interestingly, AG490, a specific inhibitor of JAK2, inhibited IL-6-induced activation of Erk1/2 as well as STAT3 (Fig. 6a). However, the IL-6-induced phosphorylation of Raf and MEK1/2 were not affected by treatment of AG490 (Fig. 7). Furthermore, AG1478, an EGFR inhibitor, was not sufficient for inactivation of IL-6-induced STAT3 in NPCs (Fig. 7). These results indicate the existences of parallel pathways promoting cell proliferation were induced by IL-6 and EGF; additionally, Erk1/2 also provided a signal bridge between the IL-6-induced JAK2/STAT3 pathway and the EGF-induced MAPKs pathway (Fig. 8).

In this study, we have evaluated the effects of IL-6 and EGF on the proliferation of adult spinal cord-derived NPCs derived from adult mouse spinal cords. To the best of our knowledge, this is the first demonstration that IL-6 and EGF stimulate proliferation of NPCs. It is also the first demonstration of how distinct signalling pathways, including JAK2/STAT3 and MAPK, play a key role in proliferation of NPCs after SCI. Additionally, a cross-talk by Erk1/2 provided a signal bridge between the IL-6-induced JAK2/STAT3 pathway and EGF- β -induced MAPK pathway. Our results clearly indicate that IL-6 and EGF induce cell proliferation, and these findings provide novel therapeutic targets for pharmacological intervention in central nervous system disease.

Figure 8. Synergy proliferation effect of NPCs by IL-6 cooperating with EGF-mediated signals. IL-6 and EGF induce the distinct intracellular signalling pathways in NPC cells. IL-6 and EGF activates STAT3 and Erk1/2, respectively. The phosphorylation of Erk1/2 via the IL-6-induced JAK2 activation contributes to the full activation of Erk1/2, leading to the proliferation of spinal cord after traumatic injury. Site of action of AG1478 and AG490 are depicted.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Figure S1. Cultural morphology and neural potencies of adult mouse spinal cord-derived neural progenitor cells (NPC) after and before differentiation. (a) Culture expanded rNPCs demonstrate small and spindle shaped morphology. Neural progenitor cells were grown as monolayers attached to PDL-coated surface or free floating neurospheres. Cultured neurospheres express several neural markers including Nestin and GFAP. Fluorescence microscopic analysis of the neurospheres expresses several neural markers. (b) After differentiation of NPCs neurosphere, differentiated NPC cells highly express motor neuron marker (NF160) and myelin basic protein (MBP) marker. Scale bar = 50 μ m. (c) RT-PCR analysis of expressed neural markers in cultured or differentiated NPC cells.

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