Jak3-dependent microgliogenesis and neurite outgrowth in spinal cord progenitors

Soo Hwan Lee, Eun Joo Baik, Sumit Barua, Jee-In Chung, A Young Kim, Soo-Yeon Lee, Soo Hwan Lee, and Eun Joo Baik

Introduction

Microglial cells are resident cells that are critical for central nervous system (CNS) development, maintenance, response to injury, and subsequent repair [1]. The microglial population also plays fundamental roles during neuronal expansion and differentiation [2]. From starting E9.5 and by 10.5 days in the mouse brain, microglial cells from myeloid precursor cells can be observed [3]. Unlike other tissue, in which myelopoiesis is restricted to the bone marrow, the precursor cells in the brain maintain the self-renewal potential [4]. Erythromyeloid precursor cells can be differentiated into ionized calcium-binding adapter molecule 1 (Iba1)-expressing cells that require transcription factor Pu.1 and interferon regulatory factor 8 (Irf8) [5]. The transcription factor Pu.1 acts as a master regulator of myeloid development. The Pu.1-deficient mice show a complete loss of microglia, indicating the pivotal role of Pu.1 in microgliogenesis [6]. Irf8 and runt-related transcription factor 1 (Runx1) are also crucial regulators of the differentiation process for microglial activation and maturation during the prenatal and postnatal period [7]. With further development, microglial cells help shape neuronal circuits during development by phagocytosing weak synapses in a developing somatosensory system [8]. Microglial cells exert some beneficial effects on spinal cord injury (SCI) in terms of neuronal repair and inflammatory resolution [9]. However, when surveilling resting microglia are activated by injury, the transformed cells can secrete neurotoxic substances that amplify the immune responses and promote phagocytic activity [10]. Although microglia play an important role in the development and pathologic conditions, the detailed regulatory pathway for their survival or activation has not yet been clarified.

The secondary mechanism of SCI that comprises inflammatory response, expanding primary lesion, and neurologically dysfunction through microglial activation is associated with the early activation of the JAK/STAT pathway [11]. In addition, modulation of the JAK-STAT pathway has the potential to affect the proliferation and differentiation of neuroprogenitors [11,12]. However, Jak kinase 3 (Jak3) may be important during the development of the CNS for astrogliogenesis and differentiation of neuroprecursor cells [12]. The role of JAK-STAT pathway after SCI begins to be concerned, and this pathway may participate in spinal tissue remodeling [11,13]. In the present study, we investigated the development of microglial cells and neurons from nestin+ progenitors.
progenitor cells spinal cord injury obtained from E13.5 spinal cord under growth factors (GF)-enriched conditions and examined whether these are associated with Jak3 signaling in the differentiation of progenitors in in-vitro SCI models.

Materials and methods

Primary spinal cord cell cultures

All animal experiments were conducted in accordance with the guidelines on the use and care of laboratory animals established by the Animal Care Committee at Ajou University. The primary spinal cord cell cultures were obtained from ICR mouse E13.5 embryos ( Orient, Seoul, Korea). Isolation from embryonic spinal cord and progenitor cultures was performed using a slightly modified method from previous study [14]. Briefly, the mice were deeply anesthetized with isoflurane and euthanized by quick cervical dislocation. Cells were then dissociated and were plated in poly-d-lysine-lamin-coated 24-well (3 × 10^6 cells/ml) plates in Dulbecco’s modified Eagle’s medium/F12 media ( Gibco, Carlsbad, California, USA), 20 ng/ml basic fibroblast growth factor (bFGF) and epithelial growth factor (EGF), and N-2 supplement (10 mM) (Invitrogen, Carlsbad, California, USA). Cells were incubated at 37°C in 5% CO2 incubators and GFs were added every third day. The cells were fixed with 4% paraformaldehyde and maintained at 4°C before immunostaining.

In-vitro scratch-induced wound model

The spinal neuroprogenitors were treated with vehicle (dimethylsulfoxide, 0.1%) and Jak inhibitor Whi-P131 (10 µM; cat: 420101, Calbiochem, California, USA) at the time of seeding. After 24 h, a scratch wound injury was made with a pipette tip (10 µl). The media were changed to remove the floating cells and treated again, and incubated at 37°C in 5% CO2 incubators and GFs were added every third day. The cells were fixed with 4% paraformaldehyde and maintained at 4°C before immunostaining.

Immunocytochemistry

For immunostaining, fixed cultured cells were blocked with 3% BSA in PBS with 0.2% Triton X-100 for 1 h and incubated overnight with primary antibodies dissolved in 0.03% BSA. We used the following primary antibodies: rabbit anti-Iba1 (1: 500; microglia marker, cat: 016-2001; Wako), mouse anti-Tuj1 (1: 1000; cat: MAB1637); and rabbit anti-actin (1: 1000; cat: A5060; Sigma). Chemiluminescent signals were acquired by scanning the membranes using the LAS-1000 system and analyzed using Multi Gauge software (Fuji Film, Tokyo, Japan).

dDNA was prepared from the total RNA as described previously [12]. Real-time PCR was performed in the CFX96 Real-Time PCR detection system using 0.5 µl of that cDNA from each sample, SYBR Green Supermix (Bio-rad), and 10 µM of each forward and reverse primer mixture. GAPDH was used as the housekeeping gene and data were quantified using the 2^-ΔΔCt method [16]. The following primers were used:

Pu.1: AGCGATGGAGAAAGCCATAG forward, CTGCA GCTCTGTGAAGTGGT reverse.

Irf8: TGGACACACAGGTTCATCC forward, CTGCT CTACCTGTGACAGAAG reverse.

CD11b: TTAATGACTCTGCGTTGCC forward, GCC CACACTAGTGTGTACAG reverse.

Runx1: TGGCACTCTGTCACCAGCAT forward, GAA GCTCTGTGCTCCTACCGGC3 reverse.

GAPDH: ACAACTTTGGCATTGTGGAA forward, GAT GCAGGAATGATGTTCTG reverse.

Statistical analysis

The data were quantified and expressed as the mean±SEM of three to six independent experiments. Analysis of variance, followed by Tukey’s post-hoc test was carried out for statistical comparisons. NIH ImageJ software was used to quantify the immunostaining data. SigmaPlot 12.0 (Systat Software Inc., San Jose, California, USA) was used to compare the groups at each time point. P values less than 0.05 were considered significant.

Results

In spinal progenitor cultures with EGF/bFGF-enriched conditions, nestin^+ cells initially proliferate as clusters...
and differentiate into cells with neurites. However, the characteristics were not maintained; instead, after days 3 and 5, the number of multipolar cells increased and clear empty spaces appeared around them (Fig. 1a, white arrow head). In contrast, the nestin$^+$ cells in a selective Jak3 inhibitor Whi-P131-treated group appeared thinner and longer and with small multipolar motile cells, but were not quantified.

To characterize the multipolar cells in the control and the vehicle-treated group, we performed double-immunostaining with nestin and Iba1. The multipolar nestin$^+$ cells were transformed into Iba1$^+$ cells. To count the number of Iba1$^+$ cells, four frames with $\times400$ magnification from each group were taken ($n=6$); $^*P<0.05$ versus the control and the vehicle. The transcription factors, Pu.1, Irf8, and Runx1 and CD11b mRNA responsible for microgliogenesis and microglial activation were measured. In the control groups, the mRNA expression increased according to days; however, Jak kinase 3 inhibitor downregulated the expression of the transcription factors. These data were obtained in duplicate for each biological preparation ($n=3$). Scale bar: 50 $\mu$m.$^*P<0.05$ versus the vehicle, $#P<0.05$ versus 0 day control. Iba1, ionized calcium-binding adapter molecule 1; Irf8, interferon regulatory factor 8; Runx1, runt-related transcription factor 1.

We investigated the changes in the mRNA levels of transcription factors, Pu.1, Irf8, and Runx1, which are responsible for microgliogenesis, microglial viability, and activation, and also measured the microglial marker CD11b mRNA. The expression of all transcription factors increased progressively after seeding; the increase in

and compared with 3 days in vitro (Fig. 1a). The Iba1$^+$ cells filled the empty spaces and they seemed to be motile and to have potent phagocytic activity (data not shown). In the vehicle-treated group, after 5 days, 8.33$\pm$1.87% of total cells were Iba1$^+$ cells, whereas 1.08$\pm$0.32% of total cells were Iba1$^+$ cells in the Jak3 inhibitors group ($P<0.05$). The nestin-Iba1-double-positive cells that were transformed into Iba1$^+$ cells from the intermediate nestin$^+$ cells were 1.01$\pm$0.12 and 1.02$\pm$0.12% of the total cells after 3 days in the control and vehicle-treated groups, and Jak3 inhibitor decreased to 0.15$\pm$0.04% of the total cells ($P<0.05$). Also, on the fifth day, the colabeled cells were completely reduced by Jak3 inhibition. Thus, Jak3 inhibition markedly reduced the numbers of Iba1$^+$ cells and also the differentiation of the nestin to Iba1 cells ($P<0.05$) (Fig. 1b).
the Pu.1, Irf8, Runx1, and CD11b mRNA after 3 days was 3.7±0.6, 3.3±0.7, 3.8±0.7, and 3.3±0.2 times in the vehicle-treated group than on day 0. Yet, Jak3 inhibition completely inhibited the increase of these transcription factors ($P<0.01$). The results indicate that Jak3 is an essential regulator of microglial viability, motility, and phagocytic function (Fig. 1c).

We used the scratch-induced wound model to observe the response of spinal progenitors to the SCI after Jak3 inhibition. The growing neurites from the edge could be observed easily. In Fig. 2a, Tuj1$^+$ neuronal fibers were present at the edge of the lesion 3 days after the injury and then the neurons were dying in the control and vehicle groups. In contrast, treatment of Whi-P131 showed more neurons with longer neurites, and these neurons could survive further. The Tuj1$^+$ fibers had crossed the midline and reached the other side of the wound 5 days after injury (Fig. 2a). The growing neurites in the control groups were observed at 3 days and did not remain at 5 days, whereas the abundant neurites in the Whi-P131 group remained after 5 days (Fig. 2a). The growing neurites from the edge to the wound area were measured by their covered area, which were 71.25±6.43 and 118.06±11.59 µm² at 3 days and 14.14±3.57 and 20.52±4.50 µm² at 5 days. However, the neurites in the Whi-P131-treated group covered significantly more area ($P<0.01$), about 519.37±46.42 and 567.35±39.18 µm² of each field, at 3 and 5 days in vitro, respectively (Fig. 2b). These results indicate that Jak3 inhibition significantly increases neurite outgrowth under a GF-enriched condition.

**Fig. 2**

Effect of Jak kinase 3 inhibitor on neurite outgrowth in a scratch-induced wound model. (A) Tuj1 (green) immunolabeled photographs represent neurite outgrowth in the scratch wound area at 3 and 5 days in vitro. In the Jak kinase 3 inhibition group, neurite outgrowth increased at 3 days and remained till 5 days. Most of the growing Tuj1$^+$ fibers crossed the midline and bridged between the two wound edges ($n=6$). (B) Upper, the skeletonized Tuj1$^+$ neurite from the wound edge at 3 and 5 days after injury. The (a′, b′, and c′) and (a″, b″, and c″) represent the frame with traced neurite, respectively, at third and fifth day in vitro. Lower, the skeletonized neurite-covered areas were quantified ($n=5$). Scale bar: 50 µm, $^*P<0.01$ versus the control and the vehicle.
Under EGF/bFGF-enriched conditions, spinal progenitor cells could differentiate into MAP2+ neurons and Jak3 inhibitor treatment could increase the number of MAP2+ mature neurons. In Fig. 3a, the number of MAP2+ cells was counted. Jak3 inhibition increased MAP2+ neurons (29.43±2.43% of the total cell) than the vehicle (10.25±2.36% of the total cell) on day 5 with long neurites (n=6, P<0.001). In Fig. 3b, Tuj1+ neurites and their soma (green) and simultaneously microglial marker Iba1+ (red) were stained. Expectedly, the multipolar cells were stained with Iba1+. Jak3 inhibition showed little Iba1+cells (Fig. 1b) and high numbers of Tuj1+ neurites (49.8±3.4% compared with the vehicle 18.4±2.1% at 3 days; 56.1±3.0% compared with the vehicle 18.3±2.8% of the total area, n=6, P<0.001). In western blotting, Tuj1 and Iba1 proteins before injury (day 0) were barely detectable. However, on day 3, Iba1 expression began to increase and was significant by day 5; in contrast, Iba1 expression was rarely detectable after Jak3 inhibition (Fig. 3c). Tuj1 expression showed the same pattern as that observed with immunostaining; it was increased on both the 3 and 5 days in vitro in the Whi-P131-treated group. These results indicate that neurite outgrowth and microglial activation were correlated inversely.

**Discussion**

Multipotential progenitors temporally expressing nestin protein are downregulated at the onset of differentiation. Embryonic nestin belongs to a class VI intermediate filament that is recognized as a sensitive marker for neuronal progenitor cells in the developing CNS [17]. Thus, expression of nestin may also implicate the neurogenesis, remodeling, and repair processes of developing and adult CNS. However, microglial repopulation after elimination of almost all microglia also occurs through a proliferation of nestin+ cells that then differentiates into microglia [18,19]. In the present study, we found that a significant proportion of nestin-expressing cells in embryonic spinal cord cells cultured in GFs-enriched can be transformed into Iba1+ microglial cells. JAK/STAT signaling activation plays a major role in SCI, which leads to the spread of neuroinflammation [20] and astrogliosis promoting glial scar formation [21]. The early JAK/STAT activation of spinal cord microglia, which occurs in peripheral nerve injury, participates in spinal tissue remodeling [13]. In our study, Jak3 signaling played critical roles in microglial activation and concomitantly decreased neurogenesis, which suggests that
the modulation of Jak3 has the potential to block neuroinflammation and aid spinal tissue repair.

In our study, a significant number of nestin+ cells transformed into the microglial cells. We observed Iba1 expression for the recognition of microglial cells; however, to show this transdifferentiation, we measured transcription factors critical for microgliogenesis including Pu.1 and Irf8. They can act simultaneously and also function subsequently for microglial development [5]. Irf8-mediated transcription can lead to the microglial phenotype with phagocytic activity [22]. Transcription factor Runx1 also regulates microglial phenotypes in prenatal and postnatal periods [7], and also even progenitor to neuron transition from undifferentiated progenitors [23]. Moreover, upregulated Runx1 expression is associated with brain injury and disease [24]. Runx1 can be a key regulator for lineage specification through their chromatin-opening activity [25], which might be associated with increased production of active ameboid microglial cells. Thus, in this study, regulation of all these transcription factors by Jak3 signaling suggests its critical role in microglial differentiation and maturation during spinal cord development.

Previously, we reported that embryonic cortical progenitors can proliferate and differentiate over time in the appropriate environment by regulation of Jak3 signaling: this downregulation of Jak3 can give rise to more Tuji+ and MAP2+ cells with long neurites [15]. In the present study, spinal progenitors showed similarity to the cortical progenitors, except that the spinal progenitors showed more survivability, had longer neurites, and higher migratory potential, although the comparison was not included in this study. In a developing cerebral cortex, microglia play a fundamental role in regulating the size of the precursor cells [26]. Also in the Xenopus spp. study, the sustained activation of the JAK-STAT pathway reduced the expression of proneurogenic genes, which normally upregulated in response to SCI [27].

In the present study, Jak3-dependent microgliogenesis under GFs-enriched conditions was accompanied by decreased neurogenesis. We could not conclude whether neuronal loss occurred before microglial activation or because of Jak3-dependent microgliosis, which might also inhibit the neurogenesis and neurite outgrowth. However, the inverse relationship between microglial activation and decreased neurogenesis was prominent. According to culture days, neuronal loss seemed to appear before microglial activation. Jak3 inhibition could increase neuronal differentiation with long neurite outgrowth and their maintenance. This was followed by increased neuronal survival and concomitantly completely attenuated microglial activation. However, Jak3-dependent microglial activation still managed to induce the breakdown of growing neurites.

Conclusion
Tyrosine kinase Jak3 is crucial for the regulation of neurite growth and microglial differentiation in the spinal cord. The more detailed mechanism of the inverse relationship between the neurite growth and microglial activation needs to be further investigated.

Acknowledgements
The authors thank Chung Yang, Cha Young Sun, MD, and Jang Hi Joo Yeu Sa Memorial Fund, Korean Research Foundation grant from the Korean government (2012R1A2A01011417), and the Chronic Inflammatory Disease Research Center (NRF-2012R1A5A2048183) for supporting our study.

Conflicts of interest
There are no conflicts of interest.

References
1 Harry GJ. Microglia during development and aging. Pharmacol Ther 2013; 139:313–326.
2 Tong CK, Vidyadaran S. Role of microglia in embryonic neurogenesis. Exp Biol Med (Maywood) 2016; 241:1669–1675.
3 Ginhoux F, Lim S, Hoeffel G, Low D, Huber T. Origin and differentiation of microglia. Front Cell Neurosci 2013; 7:45.
4 Prinz M, Erny D, Hagemeyer N. Ontogeny and homeostasis of CNS myeloid cells. Nat Immunol 2017; 18:385–392.
5 Kierdorf K, Erny D, Goldmann T, Sander V, Schulz C, Perdiguerio EG, et al. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nat Neurosci 2013; 16:273–280.
6 Satchi J, Ashauna N, Kitano S, Kino Y. A comprehensive profile of ChIP-seq-based Pu.1/Spi1 target genes in microglia. Gene Regul Syst Biol 2014; 8:127–139.
7 Zussio M, Methot L, Lo R, Greenhalgh AD, David S, Stifani S. Regulation of postnatal forebrain ameboid microglial cell proliferation and development by the transcription factor Runx1. J Neurosci 2012; 32:11285–11298.
8 Miyamoto A, Wake H, Ishikawa AW, Eto K, Shibata K, Murakoshi H, et al. Microglia contact induces synapse formation in developing somatosensory cortex. Nat Commun 2016; 7:12540.
9 DiSabato D, Quan N, Godbout JP. Neuroinflammation: the devil is in the details. J Neurochem 2016; 139:136–153.
10 Norden DM, Muccigrosso MM, Godbout JP. Microglial priming and enhanced activity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease. Neuropharmacology 2015; 96 (Pt A): 29–41.
11 Wang T, Yuan W, Liu Y, Zhang Y, Wang Z, Zhou X, et al. The role of the JAK-STAT pathway in neural stem cells, neural progenitor cells and reactive astrocytes after spinal cord injury. Biomed Rep 2015; 3:141–146.
12 Kim YH, Chung JI, Wos HG, Jung YS, Lee SH, Moon CH, et al. Differential regulation of proliferation and differentiation in neural precursor cells by the Jak pathway. Stem Cells 2010; 28:1816–1828.
13 Molet J, Mauborgne A, DioIlo M, Armand V, Geny D, Villanueva L, et al. Microglial Janus kinase/signal transduction and activator of transcription 3 pathway activity directly impacts astrocyte and spinal neuron characteristics. J Neurochem 2016; 136:133–147.
14 Langlois SD, Morin S, Yam PT, Charron F. Dissection and culture of commissural neurons from embryonic spinal cord. J Vis Exp 2010; 39:1773.
15 Pool M, Thiemann J, Bar-Or A, Fournier AE. NeuriteTracer: a novel imageJ plugin for automated quantification of neurite outgrowth. J Neurosci Methods 2008; 168:134–139.
16 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25:402–408.
17 Gilvarg A V. Nestin in central nervous system cells. Neurosci Behav Physiol 2008; 38:165–169.
18 Emorin MR, Najafi AR, Koike MA, Dagher NN, Spangenberg EE, Rice RA, et al. Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. Neuron 2014; 82:380–397.
19 Krishnasamy S, Weng YC, Thanmissetty SS, Phaneuf D, Lalancette-Hebert M, Kriz J. Molecular imaging of nestin in neuroinflammatory conditions reveals marked signal induction in activated microglia. J Neuroinflammation 2017; 14:45.

20 Dominguez E, Mauborgne A, Mallet J, Desclaux M, Pohl M. SOCS3-mediated blockade of JAK/STAT3 signaling pathway reveals its major contribution to spinal cord neuroinflammation and mechanical allodynia after peripheral nerve injury. J Neurosci 2010; 30:5754–5766.

21 You T, Bi Y, Li J, Zhang M, Chen X, Zhang K, et al. IL-17 induces reactive astrocytes and up-regulation of vascular endothelial growth factor (VEGF) through JAK/STAT signaling. Sci Rep 2017; 7:41779.

22 Masuda T, Tsuda M, Yoshinaga R, Tozaki-Saitoh H, Ozato K, Tamura T, et al. IRF8 is a critical transcription factor for transforming microglia into a reactive phenotype. Cell Rep 2012; 1:334–340.

23 Zagami CJ, Zusso M, Stifani S. Runx transcription factors: lineage-specific regulators of neuronal precursor cell proliferation and post-mitotic neuron subtype development. J Cell Biochem 2009; 107:1063–1072.

24 Wang JW, Stifani S. Roles of runx genes in nervous system development. Adv Exp Med Biol 2017; 962:103–116.

25 Lichtinger M, Hoogenkamp M, Kryspin L, Ingram R, Bonifer C. Chromatin regulation by RUNX1. Blood Cells Mol Dis 2010; 44:287–290.

26 Cunningham CL, Martinez-Cerdeno V, Noctor SC. Microglia regulate the number of neural precursor cells in the developing cerebral cortex. J Neurosci 2013; 33:4216–4233.

27 Tapia VS, Herrera-Rojas M, Larraín J. JAK-STAT pathway activation in response to spinal cord injury in regenerative and non-regenerative stages of Xenopus laevis. Regeneration (Oxf) 2017; 4:21–35.