Jak kinase 3 signaling in microgliogenesis from the spinal nestin$^+$ progenitors in both development and response to injury

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During spinal cord development, endogenous progenitors expressing nestin can migrate into the target and differentiate into neurons and other glial cells. Microglial cells can also be derived from nestin$^+$ progenitor cells, even in the adult brain. Knockdown of Jak kinase 3 (Jak3) signaling can increase neurogenesis with longer neurite outgrowth in cortical progenitor cells. This study investigated the effect of Jak3 signaling on differentiation from nestin$^+$ progenitor cells using E13.5 spinal progenitor cell cultures. In growth factors-enriched culture, developing neurons could not survive after several days and also a significant proportion of nestin-expressing cells transformed into ameboid Iba1$^+$ microglial cells, which increased exponentially after 5 days. This microgliogenesis was predominantly regulated by Jak3 signaling, which was confirmed by transcription factors responsible for microgliogenesis, and microglial migration and phagocytosis, such as Pu.1, Irf8, and Runx1. Jak3 inhibition also significantly increased the Tuj1$^+$ growing neurites with little microglial activation. These results indicated that neuronal and microglial cell differentiation was regulated primarily by Jak3 signaling and the developing neurons and neurite outgrowth might also be regulated by Jak3-dependent microglial activity. *NeuroReport* 28:929–935 Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc.

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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 neurological 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 begin 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$^+$

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 cells 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-lycine-laminin-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% CO_2 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 Jak3 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% CO_2 incubators for 3 and 5 days.

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 : 300; microglia marker, cat: 019-19741; Wako, Los Angeles, California, USA); mouse anti-Tuj1 (class III tubulin, 1 : 500; cat: MAB1637); rabbit anti-MAP2 (microtubule associated protein 2, 1 : 500; cat: AB5622); and mouse anti-nestin (1 : 500; cat: MAB353) (all from EMD Millipore, Los Angeles, California, USA). After overnight incubation, the samples were washed and incubated with secondary antibodies Alexa Fluor 488 or Alexa Fluor 594 (1 : 500; Invitrogen) for 2 h at room temperature. After staining nuclei with Hoechst33258 (Sigma; Sigma-Aldrich, Saint Louis, USA), fluorescence images were acquired.

To measure the Tuj1$^+$ neurites bridging the wound edges, the growing neurites were traced by NeuriteTracer, a multiplatform free image-processing plugin software for ImageJ (NIH, USA), as described previously [15]. After preprocessing and applying a suitable threshold, Tuj1$^+$ fibers were skeletonized, overlaid with the duplicated images using the 'image calculator' command, and areas of the growing neurites were measured by analyzing particles' function.

Western blotting, RNA isolation, and RT-PCR

Western blotting was performed as described previously [12]. Collected proteins were separated using SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts, USA). The primary antibodies that we used were rabbit anti-Iba1 (1 : 1000; cat: 016-20001; 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).

cDNA 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 pM of each forward and reverse primer mixture. GAPDH was used as the housekeeping gene and data were quantified using the $2^{-\Delta\Delta C_t}$ method [16]. The following primers were used:

Pu.1: AGCGATGGAGAAAGCCATAG forward, CTGCA GCTCTGTGAAGTGGT reverse.

Irf8: TGACACCAACCAGTTCATCC forward, CTGCT CTACCTGCACCAGAA reverse.

CD11b: TTAATGACTCTGCGTTTGCC forward, GCC CACAATGAGTGGTACAG reverse.

Runx1: TGGCACTCTGGTCACCGTCAT forward, GAA GCTCTTGCCTCTACCGC3 reverse.

GAPDH: ACAACTTTGGCATTGTGGAA forward, GAT GCAGGGATGATGTTCTG reverse.

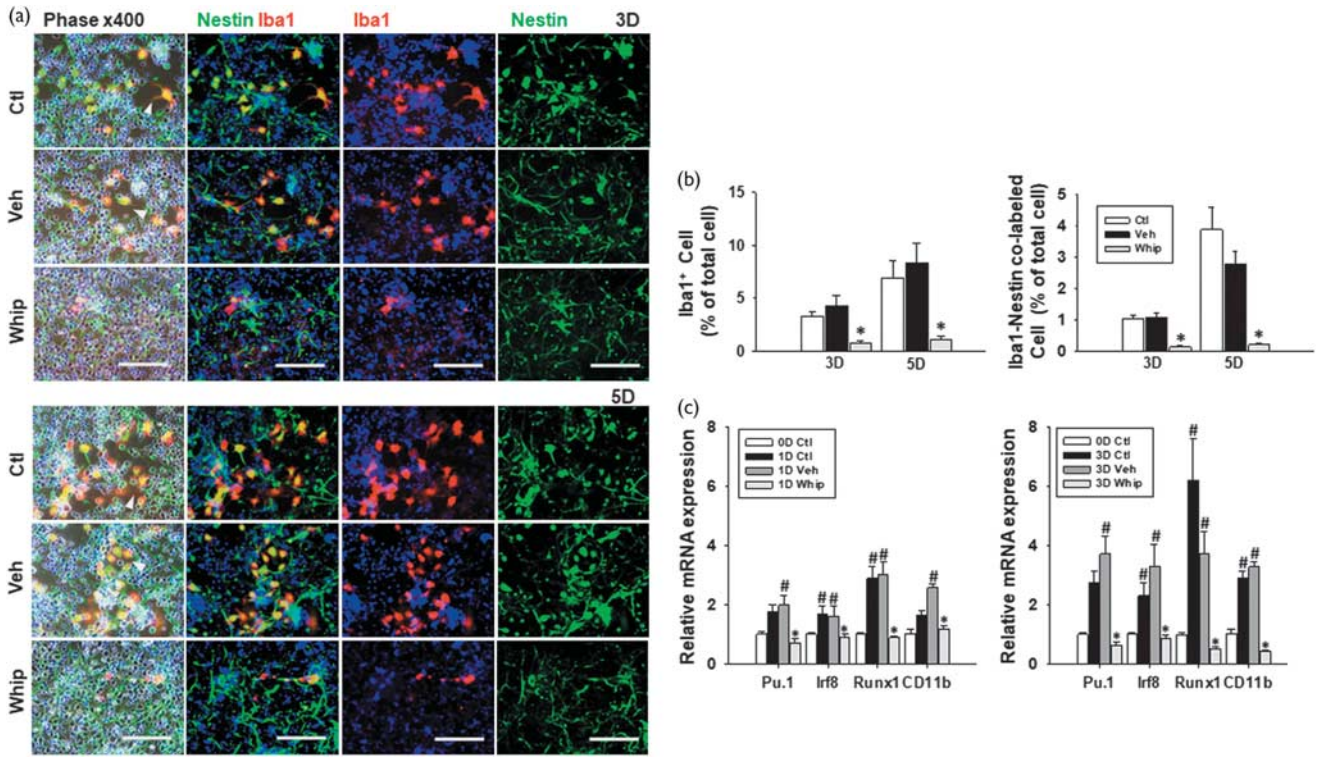
Statistical analysis

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

Fig. 1



Effect of Jak kinase 3 signaling on microglial development from nestin$^+$ cells. (a) Immunocytochemical assay stained with nestin (green) and Iba1 (red) antibodies in the spinal progenitors. In the control groups, multipolar nestin$^+$ cells were transformed into Iba1$^+$ cells. (b) The numbers of Iba1$^+$ cells and nestin-Iba1-double-positive cells were counted ($n=6$); *$P<0.05$ versus the control and the vehicle. (c) 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 μm.*$P<0.05$ 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.

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.

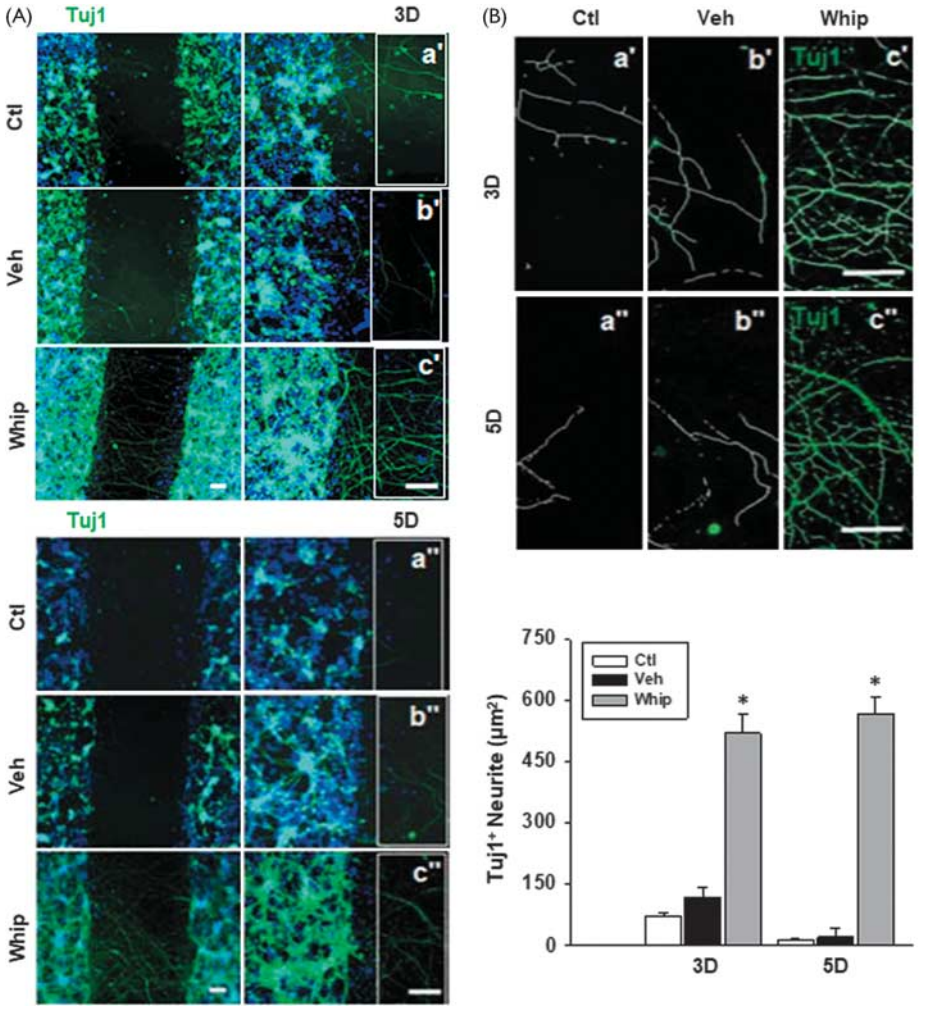
To characterize the multipolar cells in the control and the vehicle-treated group, we performed double-immunostaining with nestin and Iba1. The multipolar nestin$^+$ cells were transformed into Iba1$^+$ cells. To count the number of Iba1$^+$ cells, four frames with ×400 magnification from each group were taken ($n=6$) and the numbers of Iba1$^+$ cells were counted manually; then, the numbers were represented as the percent of the total cells. The total cells were counted from Hoechst-positive cells (450±65 cells each field). We examined whether Jak3 signaling is involved in microgliogenesis and microglial differentiation. In the control group, the numbers of Iba1$^+$ cells increased from 3.32±0.43% of total cells to 6.96±1.59% ($P<0.05$) at 5 days in vitro 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±1.87% of total cells were Iba1$^+$ cells, whereas 1.08±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±0.12 and 1.02±0.12% of the total cells after 3 days in the control and vehicle-treated groups, and Jak3 inhibitor decreased to 0.15±0.04% of the total cells ($P<0.05$). Also, on the fifth day, the cola-beled 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).

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

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).

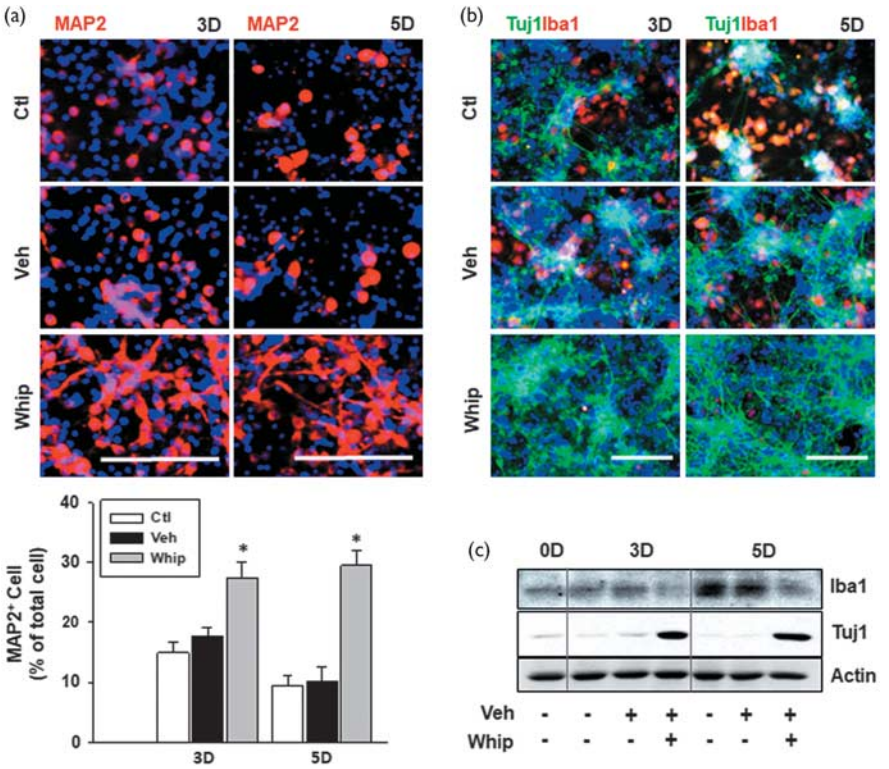
We used the scratch-induced wound model to observe the response of spinal progenitors to the SCI after Jak3 inhibition. The growing neurites from the edge could be observed easily. In Fig. 2a, Tuj1$^+$ neuronal fibers were present at the edge of the lesion 3 days after the injury and then the neurons 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 μm^2 at 3 days and 14.14 ± 3.57 and 20.52 ± 4.50 μm^2 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 μm^2 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.

Fig. 3



Inverse relationship between Tuj1 and Iba1 expression. (a) Jak kinase 3 inhibition increased MAP2$^+$ (red) neurons at 3 and 5 days. The lower graph represents the quantified MAP2$^+$ cells. (b) Photographs show the microglial Iba1$^+$ (red) and Tuj1$^+$ (green) neurites after 3 and 5 days *in vitro* ($n=5$). Tuj1$^+$ neurites were lost, and the spaces were filled with Iba1$^+$ cells. (c) In western blotting, the Tuj1 and Iba1 protein expression was also inversely regulated by Whi-P131. The blots are representative of three independent experiments. Scale bar: 50 μm. *$P<0.001$ versus the control and the vehicle. Iba1, ionized calcium-binding adapter molecule 1.

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 Tuj1$^+$ 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 proneurogenetic 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.

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Conflicts of interest

There are no conflicts of interest.

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