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Stem Cell Factor-Activated Bone Marrow Ameliorates Amyotrophic Lateral Sclerosis by Promoting Protective Microglial Migration

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Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive disease associated with motor neuron death. Several experimental treatments, including cell therapy using hematopoietic or neuronal stem cells, have been tested in ALS animal models, but therapeutic benefits have been modest. Here we used a new therapeutic strategy, bone marrow transplantation (BMT) with stem cell factor (SCF)- or FMS-like tyrosine kinase 3 (flt3)-activated bone marrow (BM) cells for the treatment of hSOD1(G93A) transgenic mice. Motor function and survival showed greater improvement in the SCF group than in the group receiving BM cells that had not been activated (BMT alone group), although no improvement was shown in the flt3 group. In addition, larger numbers of BM-derived cells that expressed the microglia marker Iba1 migrated to the spinal cords of recipient mice compared with the BMT-alone group. Moreover, after SCF activation, but not flt3 activation or no activation, the migrating microglia expressed glutamate transporter-1 (GLT-1). In spinal cords in the SCF group, inflammatory cytokines tumor necrosis factor-α and interleukin-1β were suppressed and the neuroprotective molecule insulin-like growth factor-1 increased relative to nontreatment hSOD1(G93A) transgenic mice. Therefore, SCF activation changed the character of the migrating donor BM cells, which resulted in neuroprotective effects. These studies have identified SCF-activated BM cells as a potential new therapeutic agent for the treatment of ALS.

Keywords

SOD1; microglia; BM stem cells; ALS; GLT-1

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Additional Supporting Information may be found in the online version of this article.

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by muscle weakness and bulbar paralysis caused by upper and lower motor neuron degeneration (Mitchell and Borasio, 2007). The pathogenesis of the disease is still unclear, and effective therapies to treat the disease have yet to be developed. For most patients, ALS is a sporadic disease, but about 10% of ALS have been found to harbor genetic mutations (Pasinelli, 2006). Mutations of the superoxide dismutase 1 (SOD1) gene underlie the most common type of familial ALS (Rosen et al., 1993). In addition, mutations in the transactive response DNA-binding protein-43 (TDP-43), fused in sarcoma (FUS), and optineurin genes have recently been found to be associated with ALS (Sreedharan et al., 2008; Kwiatkowski et al., 2009; Vance et al., 2009; Maruyama et al., 2010; Wang et al., 2011). Rodents with engineered SOD1 mutations are an animal model widely used to study the pathogenesis and therapy of ALS. A popular transgenic model hSOD1(G93A; SOD1-tg) contains a mutation in the SOD1 gene, which results in motor neuron degeneration and motor dysfunction (Tu et al., 1996; Matsumoto et al., 2006). The neurons of SOD1-tg mice display mitochondrial dysfunction, oxidative stress, and premature apoptosis (Magrané et al., 2012). In addition, their spinal cords exhibit marked inflammation and infiltration with large numbers of microglia and macrophages (Alexianu and Kozovska, 2001; Phani et al., 2012). Likewise, ALS patients also have an increase in infiltrating blood cells to the spinal cord (Henkel et al., 2004). Therefore, an increased inflammatory response, in particular, hematopoietic cell infiltration, appears to contribute to the pathogenesis of motor neuron dysfunction.

Different therapeutic strategies have been tested in ALS patients and in animal models of ALS. Treatment with growth factors (glial cell line-derived neurotropic factor [GDNF], human growth factor [HGF], insulin-like growth factor-1 [IGF-1], etc.) and antioxidants has been used to promote neuronal protection in ALS (Acsadi et al., 2002; Sun et al., 2002; Kaspar et al., 2003; Reyes et al., 2010; Gould and Oppenheim, 2011). Granulocyte-macrophage colony-stimulating factor (G-CSF), a known differentiation factor of hematopoietic progenitor cells, has also been tested in ALS mice (Yamasaki et al., 2010; Pollari et al., 2011). Other regenerative medicine approaches have included the use of neuronal stem cells, mesenchymal stem cells, cord blood cells, and bone marrow-derived (BM) cells as therapeutic options (Mazzini et al., 2003; Boucherie et al., 2009). For instance, wild-type BM cells and hematopoietic stem cells transplanted to SOD1 transgenic mice have been shown to preserve motor function and prolong the survival of SOD1 transgenic mice (Corti et al., 2004). Alternatively, transplantation of a population of c-kit$^+$ stem/progenitor cells from the BM produced additional therapeutic effects in ALS mice (Corti et al., 2010).

We reasoned that bone marrow transplantation (BMT) using BM cells that have been treated with a hematopoietic cell differentiation factor may be more efficacious than use of untreated BM cells alone in slowing the progression of ALS. In this study, we pretreated wild-type mouse BM cells with either stem cell factor (SCF), a c-kit ligand, or FMS-like tyrosine kinase 3 (flt3), a known hematopoietic stem cell differentiation factor, and transplanted them into SOD1-tg mice. We found that transplantation of BM pretreated with SCF produced a superior outcome compared with untreated BM or BM pretreated with flt3, as reflected by motor function preservation and survival in SOD1-tg recipient mice. Furthermore, SCF activation was found to change the character of the migrating BM cells to

a more neuroprotective phenotype by inducing the expression of GLT-1. These data suggest that transplantation of SCF-activated BM may be more efficacious than that of untreated BM in the treatment of ALS patients.

MATERIALS AND METHODS

Animals and BMT

C57BL/6, hSOD1(G93A)$^{1Gur/J}$ (SOD1-tg) and C57BL/6-Tg(UBC-GFP)$^{30Scha/J}$ (GFP mice) were purchased from Jackson Laboratories (Bar Harbor, ME). For BMT, 8-week-old female nontransgenic littermates (on a C57BL/6 background; WT) or SOD1-tg mice (on a C57BL/6 background) were irradiated (9 Gy) and injected with 4 million BM cells isolated from male WT (nontransgenic littermates on C57BL/6 background), SOD1-tg (on C57BL/6 background), or GFP mice (on C57BL/6 background) as we previously described (Brevet et al., 2010). For studies in which BM cells were preincubated with differentiation factors, whole bone marrows were incubated with stem cell span media (Stem Cell Technologies, Vancouver, British Columbia, Canada) containing SCF (100 ng/ml; R&D Systems, Minneapolis, MN), media containing flt-3 (100 ng/ml; R&D Systems), or stem cell span media alone for 12 hr at 37°C. After preincubation with differentiation factors, BM cells were washed with 0.1 M PBS, checked for cell viability with Trypan blue (Sigma, St. Louis, MO), and injected into recipient mice through the tail vein. All animals were housed and provided with water and mouse chow ad libitum and maintained under a 12-hr light and dark cycle. All animal experimental protocols were approved by the Institutional Animal Care and Usage Committee (IACUC) at Shiga University of Medical Science and were performed according to the guidelines of the IACUC at Shiga University of Medical Science.

Behavior Test and Evaluation of Survival

Rota-Rod tests (Ugo Basile, Comerio-Varese, Italy) were performed once per week from 1 week before BMT to physiological death (when the result of Rota-Rod tests showed “0 second,” we judged the condition of the mice as physiologically dead). Rota-Rod tests were performed at a range from 5 rpm/min to a maximum of 50 rpm/min for 5 min (acceleration was 9 rpm/min^2). The averages of three medians of five times trial for each mouse with an intertrial interval of at least 3 min were calculated and used for analysis. For the Kaplan-Meier survival curve, the number of living mice was counted using the definition of physiological death until all mice were recognized as such.

Immunohistochemical Analysis

Sections of the spinal cord from each mouse obtained after transcardiac perfusion and fixation were incubated with the following antibodies: anti-CD68 antibody (rat monoclonal; BioLegend, San Diego, CA), anti-c-kit antibody (rabbit polyclonal; Cell Signaling, Danvers, MA), anti-M-CSFR antibody (rabbit polyclonal; Cell Signaling), anti-MAP2 antibody (rabbit polyclonal; Chemicon, Temecula, CA), anti-GFAP antibody (rabbit polyclonal; Promega, Madison, WI), anti-Iba1 antibody (rabbit polyclonal, Wako, Osaka, Japan), and anti-GLT-1 antibody (rabbit polyclonal; Cell Signaling) at 4°C overnight. After incubation with the primary antibodies, the sections were incubated with species-matched fluorescence-labeled secondary antibodies (goat anti-rat Alexa Fluor 555, goat anti-rabbit Alexa Fluor

555, and goat anti-rabbit Alexa Fluor 633; Molecular Probes, Eugene, OR). Sections were then mounted with Vectashield mounting medium with or without DNA staining using 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). All sections were observed under a confocal laser microscope (C1si; Nikon, Tokyo, Japan) with EZ-C1 3.90 software (Nikon). The quantitative analysis of immunoreactive fluorescent densities for anti-CD68 antibody, anti-c-kit antibody, and anti-M-CSFR antibody was measured in ImageJ (v. 1.44p; National Institutes of Health). The positive densities were subtracted, and background fluorescence was divided by each visual field area. A mean density value was determined by sampling from eight to 10 scenes for each individual. For quantitative analysis of the population of Iba1-positive cells, the numbers of cells were counted in each single visual field (400 × 400 μm) and determined by sampling from eight to 10 sections for each individual.

Histological Analysis of Surviving Motor Neurons in Anterior Horn of Spinal Cord Sections

Spinal cord sections from each mouse were incubated with NeuroTrace 435/455 (blue fluorescent Nissl stain; Molecular Probes) for Nissl staining and H&E staining. The sections were observed under a confocal laser microscope and a light microscope. The cells that had a positive cytosolic staining of Nissl were counted as motor neurons in the bilateral anterior horns of spinal cords, because all neurons in anterior horns should be motor neurons, as previously described (Boucherie et al., 2009). The mean number of motor neurons in bilateral anterior horns of spinal cord was determined by sampling from eight to 10 sections for each individual. The morphology of neurons was observed with both Nissl and H&E stainings. The blue color of Nissl stain was converted to green by the EZ-C13.90 software (Nikon) for the visualization of pictures.

Histological Analysis of GFP Expression in the Spinal Cord

For GFP expression analysis, spinal cord sections from each mouse were made and mounted with Vectashield mounting medium with or without DAPI. Sections were then observed by confocal laser microscopy, and we counted the number of GFP-positive cells in whole spinal cord sections. The mean number of GFP-positive cells in spinal cord was standardized by each single visual field (400 × 400 μm) and determined by sampling from eight to 10 sections for each individual.

Western Blotting

Spinal cord tissues were lysed with RIPA buffer (150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl buffer, pH 7.4). The lysates were centrifuged (12,000*g*) for 20 min at 4°C. After the protein concentrations were determined by using a Bradford assay (Bio-Rad, Hercules, CA), 20 μg of protein from the supernatants was separated by 7.5–15% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride filter (Immobilon Millipore, Bedford, MA) and then incubated overnight at 4°C with an anti-GLT-1 antibody (rabbit polyclonal; Cell Signaling), an anti-GFP antibody (mouse monoclonal; Chemicon), or an anti-β-actin antibody (mouse monoclonal; Sigma, St. Louis,

MO). The membranes were incubated in a 1:2,000 dilution of either a horse-radish peroxidase-conjugated anti-rabbit immunoglobulin or an anti-mouse immunoglobulin (Bio-Rad) for 1 hr at room temperature. Antibody binding was detected by incubating the blots in enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) and exposing them to Kodak X-omat autoradiography film (Eastman Kodak Company, Rochester, NY). The intensity of the immunoblot bands was analyzed in ImageJ software. All data were normalized to β-actin.

Quantitative RT-PCR Analysis

Total RNA in spinal cord tissues from each mouse was extracted with an RNeasy Kit (Qiagen, Valencia, CA) and treated with DNase I (Life Technologies) digestion. After reverse transcription using oligo dT primer (Life Technologies), each mRNA expression level was quantified by real-time PCR using a LightCycler 480 (Roche Diagnostics, Manheim, Germany) with the SYBR green method. The following primers were used: GLT-1, forward primer 5-TAACTCTGGC GGCCAATGGAAAGT-3 and reverse primer 5-ACGCTGG GGAGTTTATTCAAGAAT-3; Tnfα, forward primer 5-CACGTCGTAGCAAACCACCAAGTGG-3 and reverse primer 5-GATAGCAAATCGGCTGACGGTGTGG-3; iNOS, forward primer 5-TTGGAGCGAGTTGTGGATTG-3 and reverse primer 5-GTAGGTGAGGGCTTGGCTGA-3; IL-6, forward primer 5-ACGGCCTTCCCTACTTCACA-3 and reverse primer 5-CATTTCCACCATTTCCCAGA-3; IL-10, forward primer 5-TGGACAACATACTGCTAACCGAC-3 and reverse primer 5-CCTGGGGCATCACTTCTACC-3; IL-1β, forward primer 5-CAACCAACAAGTGATATT-3 and reverse primer 5-GATCCACACTCTCCAGCTG-3; IGF-1, forward primer 5-CTGAGCTGGTGGATGCTCT-3 and reverse primer 5-CACTCATCCACAATGCCTGT-3; and βactin, forward primer 5-CGTGCGTGACATCAAAGAGAA-3 and reverse primer 5-TGGATGCCACAGGATTCCAT-3. The results were analyzed in LightCycler 480 software, version 1.5 (Roche Diagnostics). All data were normalized to β-actin. The results from each treated SOD1-tg mouse were evaluated as the ratio against the results from nontransgenic littermates.

Statistical Analysis

All data are expressed as means ± SD. In vivo analyses were performed with five to eight mice/group unless otherwise specified. For multiple data sets, one-way ANOVA and Scheffe's test were used. The log rank test was used for statistical analysis of the Kaplan-Meier curve. Data were considered significant at $P < 0.05$.

RESULTS

SCF Enhances the Therapeutic Effect of BMT on Motor Function and Survival in Sod1-Tg Mice

BM cells that had been preincubated for 12 hr with SCF (WT-SOD1 + SCF group) or flt3 (WT-SOD1 + flt3 group) or sham incubated BM (WT-SOD1 group), were transplanted into

8-week-old SOD1-tg mice (Fig. 1A). After BMT, motor function and survival were monitored until the mice were recognized as physiologically dead ("physiological death" as defined in Materials and Methods) and compared with those in SOD1-tg with no BMT (SOD1) and in SOD1-tg mice transplanted BM cells from SOD1-tg mice with no preincubation (SOD1-SOD1 group; Fig. 1B,C). We found that transplantation of SCF-activated BM (WT-SOD1 + SCF) significantly improved motor function and prolonged the survival of the SOD1-tg mice. In contrast, these parameters remained unchanged in the WT-SOD1 + flt3 group compared with the WT-SOD1 group (Fig. 1B,C). In parallel studies, we found that transplantation of BM from GFP mice led to improved motor function in SOD1-tg mice (Supp. Info. Fig. 1). Similarly, the WT-SOD1 group also showed a trend toward improved motor function and a better survival rate compared with the SOD1-SOD1 group. However, BMT using SCF activated BM greatly enhanced the therapeutic effects of BMT compared with nonactivated BM cells (WT-SOD1 group; Fig. 1B,C).

SCF-Activated BM Cells Reduce Motor Neuron Degeneration in Sod1-Tg Mice

We next quantified the number of surviving motor neurons in the anterior horns of spinal cords from each treatment group (SOD1-SOD1, WT-SOD1, WTSOD1 + SCF, or WT-SOD1 + flt3 groups) at 16–20 weeks old by using Nissl staining and found significantly more motor neurons in the WT-SOD1 (nonactivated) group than in the SOD1-SOD1 group (Fig. 2). In addition, the number of Nissl-positive motor neurons in the WT-SOD1 + SCF group was higher than that in the WT-SOD1 group, and a higher number of motor neurons survived in the WT-SOD1 + SCF group compared with the other treatment groups (Fig. 2B). In contrast, the number of motor neurons in the WT-SOD1 + flt3 group was similar to that in the WT-SOD1 group (Fig. 2B). Moreover, the morphology of neurons in the WTSOD1 + SCF group was better preserved compared with other groups and was very similar to the morphology of neurons seen in the spinal cords of the nontransgenic littermates (WT group, which had not received BMT, used as control [Fig. 2A] and by H&E staining [data not shown]).

Transplanted BM-Derived Cells Home to the Spinal Cord and Have the Characteristics of Microglia in Sod1-Tg Mice

To analyze the mechanisms by which pretreatment with SCF affected the characteristics of the transplanted BM cells in the spinal cord in SOD1-tg mice, we performed a BMT experiment using GFP donor BM cells. Isolated BM cells from adult wild-type GFP mice were incubated with SCF or flt3 or in media alone (SCF-BMT, flt3-BMT, BMT, respectively) for 12 hr and were then transplanted into 8-week-old SOD1-tg mice (Fig. 3A). At 8–12 weeks after BMT, the spinal cords were isolated from the mice in each group. It was possible that SCF activation changed the character of BM-derived cells or induced the differentiation of BM cells into other cell types such as neurons, astrocytes, or microglia, so we performed immunohistochemical analyses of spinal cord sections from SOD1-tg mice that had received GFP BM cells and stained the sections with MAP2 (neuron marker), GFAP (astrocyte marker), or Iba1 (microglia marker; Fig. 3B). Many GFP-positive cells had diffusely homed to the spinal cords in the SCF-BMT group; most of these cells were positive for Iba1, but few were positive for MAP2 or GFAP (Fig. 3B). A similar pattern of marker staining was observed in the flt3-BMT and BMT groups (data not shown). However,

the population of Iba1-positive cells with GFP signals in the SCF-BMT group was much higher than in the flt3-BMT and BMT groups, whereas the population of endogenous Iba1-positive cells (without GFP) was not different among the three groups (Fig. 3C). These results suggest that the recruitment of cells from the BM to the spinal cord under disease conditions consists of microglia-like cells but not neurons or astrocytes.

SCF-Activated BM Cells Promote the Migration of Microglia to the Spinal Cord in Sod1-Tg Mice

In 16-week-old SOD1-tg mice, many CD68 (a monocyte-macrophage lineage marker)-positive cells were diffusely distributed in the spinal cord sections. Cells expressing c-kit (a receptor for SCF) and macrophage colony-stimulating factor receptor (M-CSFR; a BM-derived progenitor cell-related maker) were also scattered diffusely in the spinal cord sections taken from SOD1-tg, but no such positively staining cells were observed in spinal cord sections from the wild-type mice (WT group, which had not received BMT, used as control; Supp. Info. Fig. 2). In addition, cells that were immunopositive for CD68, c-kit, and M-CSFR were markedly increased in the spinal cord sections after BMT of cells from wild-type GFP mice to SOD1-tg mice, and most of these cells expressed GFP signals after transplantation (thus were of BM origin; Supp. Info. Fig. 3). Quantification of the fluorescence intensities of CD68, c-kit, and M-CSFR in the spinal cord of SOD1-tg mouse BMT recipients revealed greatly increased level of cells expressing these markers after BMT from SOD1-tg donors (SOD1-SOD1 group) compared with the wild-type mice (WT group, which had not received BMT, used as control; Supp. Info. Fig. 4). In SOD1-tg mice after BMT from GFP mice (GFP-SOD1 group), the expression of CD68, c-kit, and M-CSFR was two to five times higher than that in the SOD1-SOD1 group (Supp. Info. Fig. 4). The migration of BM-derived cells was enhanced by both disease conditions and BMT in the wild-type mice. Next, to investigate the effect of SCF on the migration of BM-derived cells, we compared the GFP-positive cell number in the spinal cords among the three BMT groups including BMT using untreated BM from GFP mice (BMT group), SCF-activated BM from GFP mice (SCF-BMT), and flt3-activated BM from GFP mice (flt3-BMT; Fig. 4). At 8–12 weeks after BMT, the spinal cord was isolated from the three groups, fixed, and observed under a confocal microscope for GFP signal. The results indicated that the GFP-positive cells were most abundant in the SCF-BMT group (Fig. 4A), at a level 1.6–1.8 times higher than in the other two groups (Fig. 4B). The difference between the population of GFP-positive cells in the SCF-BMT group and that of the other two groups was likely to be consistent with the balance of the Iba1-positive cells with GFP between the SCF-BMT group and the other two groups (Figs. 3C, 4B). Similar results were obtained when the GFP levels in the spinal cord homogenates were evaluated by Western blotting; the expression of immunoreactive GFP protein in the SCF-BMT was again the highest among the three groups (Fig. 4C,D). These results suggest that SCF activation promotes the migration of BM cells to the spinal cord in SOD1-tg mice, which are mainly microglia.

SCF Activation of BM Induces Expression of GLT-1 in Transplanted BM-Derived Microglia in the Spinal Cord of Sod1-Tg Mice

We next evaluated the protein and mRNA expression of GLT-1 in the spinal cord of 16–20-week-old SOD1-tg mice after BMT with SCF-activated, flt3-activated, or nonactivated BM.

GLT-1 protein and mRNA expressions in the whole spinal cord were highest in the SCF-BMT group (Fig. 5A–C). Immunohistochemical analysis of GLT-1 protein expression indicated that the GFP-positive cells expressed GLT-1 in spinal cord tissues in the SCF-BMT group (Fig. 5D). To determine the type of cells that expressed the GLT-1 protein, we costained the sections simultaneously for both GLT-1 and GFAP (Fig. 6). In the BMT and flt3-BMT groups, the GFAP-positive cells were astrocytes, which were the main GLT-1-positive cells. Few GFP-positive cells were found to coexpress GLT-1, indicating that BM-derived microglia did not express GLT-1 in the BMT and flt3-BMT groups. In contrast, in the SCF-BMT group, GFP-positive cells expressed GLT-1, but similar numbers of GFAP-positive and GFP-negative cells (endogenous astrocytes) were found to express GLT-1 as well as in the BMT and flt3-BMT groups (Fig. 6). Migrated microglia (GFP-positive cells) expressed GLT-1 only in the spinal cords of the SCF-BMT group at 16–20 weeks. These results indicate that SCF activation changes the characteristics of BM-derived microglia to a GLT-1-expressing phenotype.

SCF Activation of BM Alters the Expression of Neurotoxic and Neuroprotective Molecules in the Spinal Cord of Sod1-Tg Mice

Next, quantitative RT-PCR was performed to investigate the other neuroprotective effects by the SCF activation in spinal cord from each treatment group of SOD-tg mice. The mRNA expressions of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) were quantified as neurotoxic molecules and those of inducible nitric oxide synthase (iNOS), interleukin-10 (IL-10), and insulin-like growth factor 1 (IGF-1) were quantified as anti-inflammatory or neuroprotective molecules (Fig. 7). In neurotoxic inflammatory markers, the mRNA expression of TNF-α was suppressed in the SCF-BMT group less than in the other groups (Fig. 7A). In addition, the expression of IL-1β in SCF-BMT group was lower than in non-BMT and in flt3-BMT groups, although IL-6 expression was similar among all SOD-tg groups (Fig. 7C,E). For neuroprotective molecules, the expression of IGF-1 was higher in the three BMT groups than in the non-BMT SOD-tg group (Fig. 7F), but the expression of iNOS and IL-10 was not changed among all SOD-tg groups (Fig. 7B,D). These results indicate that SCF activated BM is effective to protect from the disease through not only GLT-1 but also other molecules such as TNF-α, IL-1β, and IGF-1.

DISCUSSION

The results of this study indicate that SCF-activated BMT further enhances the effect of BMT from nontransgenic mice on ALS disease progression and survival. After SCF-activated BMT, the number of BM-derived cells that homed to the spinal cord increased markedly where they expressed microglia marker. Moreover, BM-derived microglia also expressed GLT-1, a protective molecule for neurons. In addition, the expression of inflammatory cytokines such as TNF-α and IL-1β was suppressed and the expression of IGF-1, a neuroprotective molecule, was increased compared with the non-BMT SOD-tg mice. Therefore, activation with SCF enhances the migration of microglia and promotes neuroprotection by alteration of GLT-1, TNF-α, IL-1β, and IGF-1 expression.

Effect of Growth Factors and Differentiation Factors on ALS

Previous reports have shown that several kinds of growth factors including IGF-1 and BDNF, or progenitor cell differentiation factors such as G-CSF and c-kit, are protective against ALS (Kaspar et al., 2003; Corti et al., 2010; Yamasaki et al., 2010; Gould and Oppenheim, 2011; Henriques et al., 2011; Pollari et al., 2011). This is due to the fact that growth factors directly affect neurons or neuronal tissues and suppress disease progression (Yamasaki et al., 2010; Gould and Oppenheim, 2011; Pollari et al., 2011). However, it has also been reported that G-CSF, c-kit, and SCF stimulate progenitor cells including neuronal stem cells, hematopoietic stem cells, and mesenchymal stem cells, to differentiate into other cell types (Galli et al., 1994; Piao et al., 2012). In particular, studies have shown that the progenitor cells can transdifferentiate following SCF treatment to acquire neuronal characteristics (Jin et al., 2002). In this study, we hypothesized that BM-derived cells might differentiate into neurons or astrocytes after activation with SCF and flt3, which are differentiation factors for BM cells. However, we found that GFP-positive (BM-derived) cells expressed microglial markers but not neuronal or astrocyte markers. Furthermore, Olig2 as the marker of oligodendrocyte was not stained in GFP-positive cells as well (data not shown). This result suggests that SCF treatment affects the migration and character of microglia but does not induce the transdifferentiation of BM cells to neurons or astrocytes. The effect of SCF administration on BM cells in vitro before transplantation was very different from the effect of the systemic injection of SCF. Considering that the SCF effect endured for long time after BM cells were incubated with SCF for only 12 hr, SCF might work in determination of the direction of cell differentiation, because SCF stimulation was transient, not continuous. Indeed, SCF induced the differentiation of monocyte-macrophage lineage cells (Zhang and Fedoroff, 1998). Moreover, cytokine production and chemotaxis were induced downstream of SCF stimulation (Zhang and Fedoroff, 1998). Therefore, we suspected that BM cells activated by SCF were transdifferenciated into microglia and programmed to be easy to migrate to inflammatory lesions, such as those in the spinal cord in ALS. However, to clarify the mechanism in detail, additional future experiments will be necessary and are expected.

Function of Microglia in Neurodegenerative Disease

Blood cells appear in neuronal tissues in many kinds of neuronal disease (Wekerlea et al., 1986; Bradl, 2003). For instance, white blood cells have been observed in the neuronal tissues of various immune diseases, such as multiple sclerosis and Guillain-Barre syndrome (Giovannoni, 1996). However, recent studies have shown that several kinds of white blood cells migrate to neuronal tissues in degenerative disease. Microglia have also been shown to be involved in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and ALS (Henkel et al., 2004; Minghetti, 2005), for which they have been reported to have a dual function in neuroprotection and neurotoxicity (Liao et al., 2012). In some reports, nonneuronal cells, including microglia and astrocytes, which express mutant SOD1 were neurotoxic; in contrast, wild-type microglia and astrocytes were neuroprotective (Clement et al., 2003; Fendrick et al., 2007; Xiao et al., 2007). In our study, mutant SOD1 BM cells migrated to the spinal cord less than wild-type BM cells, and did not show neuroprotective effects. These results were consistent with previous report that the microglia expressing mutant SOD1 showed accumulation of superoxide, which resulted in

dysfunction and low response against inflammation (Xiao et al., 2007). Furthermore, activated by SCF, a number of microglia migrated to the spinal cord under ALS disease conditions. The number of microglia and their phenotype were altered by SCF activation compared with untreated BMT. The microglia expressed GLT-1 and exhibited a neuroprotective phenotype, a phenomenon consistent with previous reports (Nakajima et al., 2008; Persson and Rönnbäck, 2012). SCF stimulation of BM cells may have the potential to change the phenotype of microglia from neurotoxic to neuroprotective.

Efficacy of SCF Activation Against BM Cells and Microglia Migration

SCF is the ligand for c-kit and a proliferation factor for hematopoietic stem cells (Hoffman et al., 1993) and has been reported to stimulate neurogenesis in vivo and in vitro (Jin et al., 2002). However, in this study, SCF activation promoted BM cells to transdifferentiate into microglia. Modulation of microglia by SCF has been reported. SCF is related to microglial survival and involved in neuron–microglia interactions (Zhang and Fedoroff, 1998). Furthermore, SCF enhances the microglial mRNA expression of NGF, BDNF, and CNF and downregulates microglial expression of the inflammatory cytokines TNF-α and IL-1β (Zhang and Fedoroff, 1998). Our results also showed the downregulation of TNF-α and IL-1β in spinal cord by SCF-activated BM.

In other disease models, the administration of SCF has been used as a treatment in a mouse stroke model and has achieved neuroprotective effects (Kawada et al., 2006; Zhao et al., 2012). In addition, combination therapy using SCF and G-CSF reduced β-amyloid deposits in the brains of mice in an Alzheimer's disease model and was associated with decreased progression of the disease (Li et al., 2011). Circulating BM-derived cells in this model had an increased number of augmented BM-derived microglia cells in the brain (Kawada et al., 2006). This phenomenon is quite similar to our results, which showed that GFP-positive microglia migrate to the spinal cord, where they display a neuroprotective effect. Consistent with our findings, SCF has been reported to be involved in the mobilization of BM cells in tissues (Galli et al., 1994). However, the chronic administration of G-CSF continuously elevates neutrophilic granulocytes in the blood, which has been shown in leukopenia patients under immunosuppressant drugs in the clinical setting (Moore, 1991; Yamasaki et al., 2010; Henriques et al., 2011). Likewise, it is possible that neutrophilic granulocytes may be elevated by systemic injection of SCF in vivo. In this study, the differentiation factors were not directly injected into animals but were preincubated with BM cells before transplantation, ensuring decreased systemic effects of the growth factor treatment. Further studies using SCF and G-CSF should weigh the potential side effects of systemic administration of growth factors against possible therapeutic benefits. The safe use of these molecules has already been demonstrated in the treatment of stroke, Alzheimer's disease, ALS, and other neuronal diseases (Yamasaki et al., 2010; Li et al., 2011; Zhao et al., 2012), for which the therapeutic effects are dependent on the appropriate dose, frequency, and route of administration of the factors.

Clinical Relevance of SCF-Activated BMT for ALS

Recently, stem cell transplantation has been proposed as a new therapy for ALS patients (Mazzini et al., 2003), and hematopoietic stem cell transplantation has already been

performed in patients with sporadic ALS (Appel et al., 2008). In the latter study, CD68- or CD1a-positive cells derived from a donor were shown to home to pathological sites of spinal cord injury, although no clinical benefits were observed. Another group demonstrated a new approach for transplantation, in which mesenchymal stem cells were directly transplanted into the spinal cord (Mazzini et al., 2010). The study confirmed the feasibility and safety of this invasive method and provided some evidence that motor neurons were preserved around the spinal cord at the level of stem cell injection, although mortality was not affected. Despite the progression of stem cell therapies, therapeutic efficacy as defined by clinical remission or reversal has yet to be achieved. Here we have demonstrated a new approach in which BM cells are preincubated with growth factors prior to transplantation. Our results indicate that pretreatment of BM cells with SCF improves treatment efficacy. SCF treatment of BM cells prior to transplantation has been reported to improve the antileukemic activity of the transplanted cells, because SCF improves the yield of hematopoietic progenitor cells and enhances the immunotherapeutic potential of allografts (Hartung et al., 2003). Here we have shown that a large number of BM-derived cells migrated to the spinal cord in the SCF-BMT group. Moreover, the cells that migrated there were found to express GLT-1 (Persson and Rönnbäck, 2012). At the same time, SCF activation promoted the neuroprotection by alteration of TNF-α, IL-1β, and IGF-1 expression. This phenomenon is analogous to that observed in the treatment of leukemia, in which SCF activation affects the differentiation fate of BM cells. We conclude that the differentiation factor SCF enhances the therapeutic potential of BM cells in a mouse ALS model and suggest that transplantation of SCF-activated BM cells may be a promising new strategy for the treatment of ALS patients.

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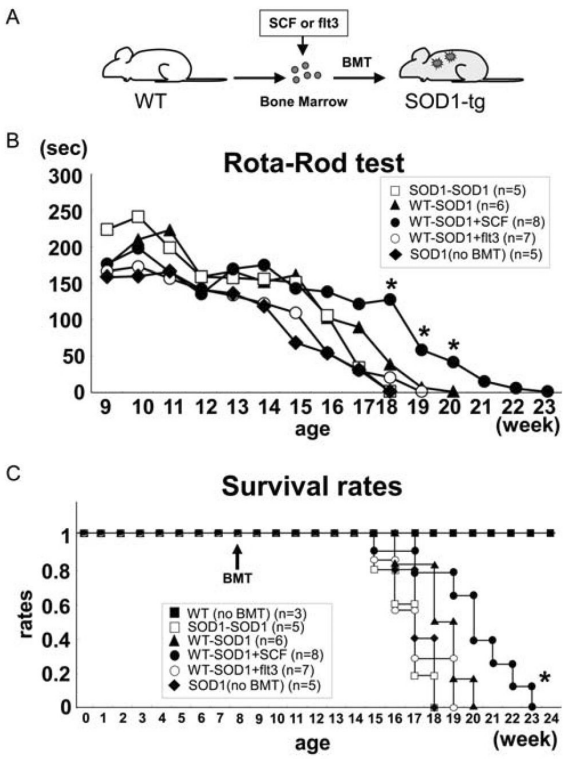

Fig. 1.

Motor function test (Rota-Rod test) and survival rates of SOD1-tg mice after transplantation with SCF- or flt3-activated bone marrow (BM) cells from nontransgenic littermates (wild-type mice; WT). **A:** Scheme of BM transplantation (BMT) from WT to SOD1 transgenic mice (SOD1-tg). BM cells isolated from WT were treated with SCF or flt3 for 12 hr before being transplanted into SOD1-tg mice. **B:** Rota-Rod tests of SOD1-tg mice (no BM transplantation, SOD1 (n = 5; lozenges) and SOD1-tg mice that received BM cells from SOD1-tg mice (SOD1-SOD1; n = 5; open circles) or from WT that were treated with no growth factors (WT-SOD1; n = 6; triangles), with SCF (WT-SOD1 + SCF (n = 8; solid circles) or with flt3 (WT-SOD1 + flt3 (n = 7; open circles) every week for 10–15 weeks after BMT at 8 weeks old. Each plot shows the average hanging time in the Rota-Rod test for each mouse. ★$P < 0.05$. **C:** Survival rates of WT mice (n = 3; solid squares), SOD1-tg mice (SOD1; n = 5; lozenges), and SOD1-tg mice transplanted with BM cells from SOD1-tg mice (SOD1-SOD1; n = 5; open squares) or from WT that were treated with no growth factors (WT-SOD1; n = 6; triangles), with SCF (WT-SOD1 + SCF (n = 8; solid circles), or with flt3 (WT-SOD1 + flt3 (n = 7; open circles). ★$P < 0.05$ vs. all other SOD1 groups.

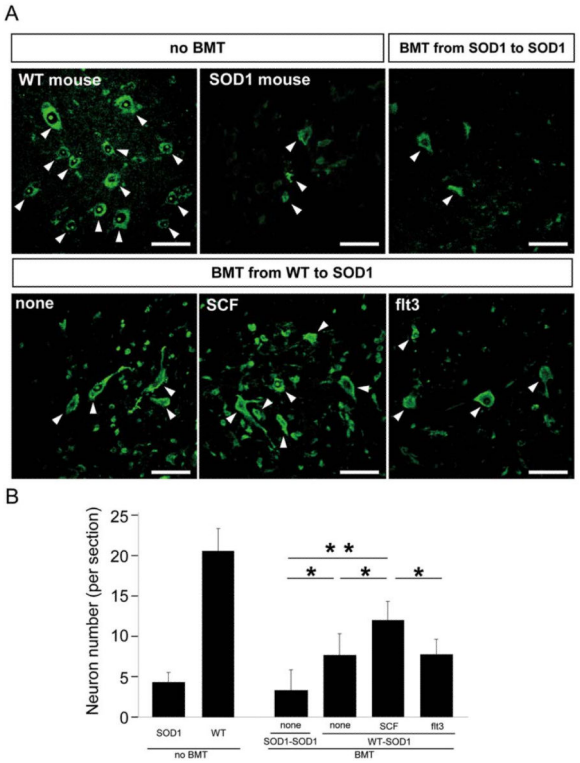

Fig. 2.

Nissl staining of spinal cord sections in SOD1-tg mice after transplantation with SCF- or flt3-activated bone marrow (BM) cells from nontransgenic littermates (WT). **A:** Nissl stain (green) in the hemianterior horns of spinal cord sections in the non-BM transplantation (BMT) control (no BMT) from WT and SOD1 transgenic (SOD1-tg) mice (upper left two); the BMT groups from SOD1-tg (upper right); and the BMT groups from WT with no growth factors (lower left), with SCF and with flt3 (lower right two). Arrowheads showed motor neurons. **B:** Quantitative analysis of the number of surviving motor neurons stained with Nissl in the bilateral anterior horns of spinal cord sections from two no-BMT control groups and four BMT groups (n = 5 in each group). The neuronal cell number of Nissl-positive staining was counted per each section. Bars show mean ± SD. ★$P < 0.05$, ★★$P < 0.01$. Scale bars = 50 μm.

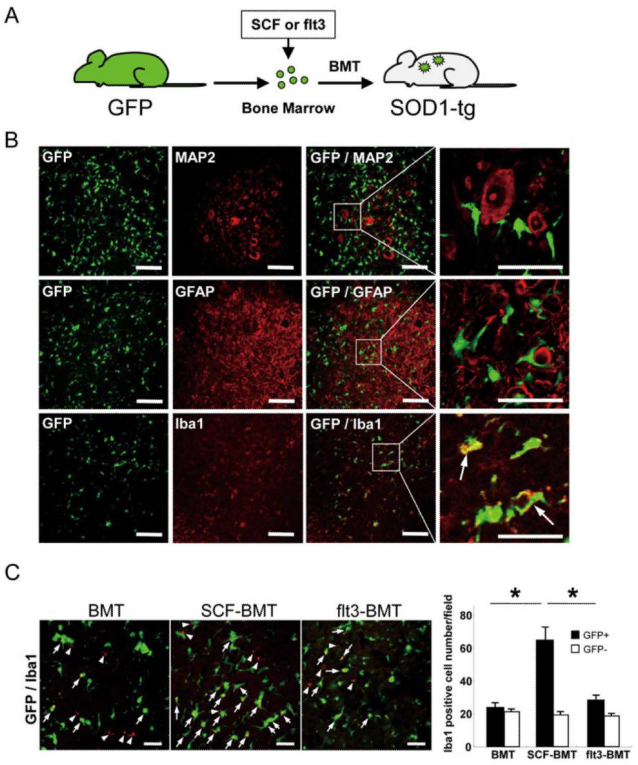

Fig. 3.

Characteristics of GFP-positive cells of spinal cords in SOD1-tg mice after BM transplantation (BMT) from GFP mice. **A:** Scheme of BMT from GFP transgenic mice (GFP) to SOD1 transgenic mice (SOD1-tg). BM cells from GFP mice were treated with SCF or flt3 for 12 hr, after which they were transplanted into SOD1-tg mice. **B:** Immunohistochemical analysis with anti-MAP2 (red, upper panels), anti-GFAP (red, middle panels), and anti-Iba1 (red, lower panels) were performed on spinal cord sections from SOD1-tg mice after transplantation of SCF-activated BM cells from GFP transgenic mice. Leftmost panels show GFP signals (green), the second panel from the left shows each of the markers (red), and the third panel from the left shows the merged (GFP signal and marker signal) images. The boved areas are enlarged at right. Arrows show merged cells (yellow) with GFP and Iba1. **C:** Quantitative analysis of the number of Iba1-positive cells (red) with GFP (green, GFP^+) or without GFP (GFP^-) signals in spinal cord sections from SOD1-tg mice after BMT of GFP-positive BM treated with no growth factor (left, BMT), with SCF (middle, SCF-BMT), or with flt3 (right, flt3-BMT) for 12 hr. Arrows show Iba1-positive cells with GFP, and arrowheads show Iba1-positive cells without GFP. The numbers of Iba1-positive cells were standardized by single visual field (400 × 400 μm). Bars show mean ± SD. ★$P < 0.01$. Scale bars = 100 μm in the three leftmost panels in B; 50 μm in the rightmost panels in B; 25 μm in C.

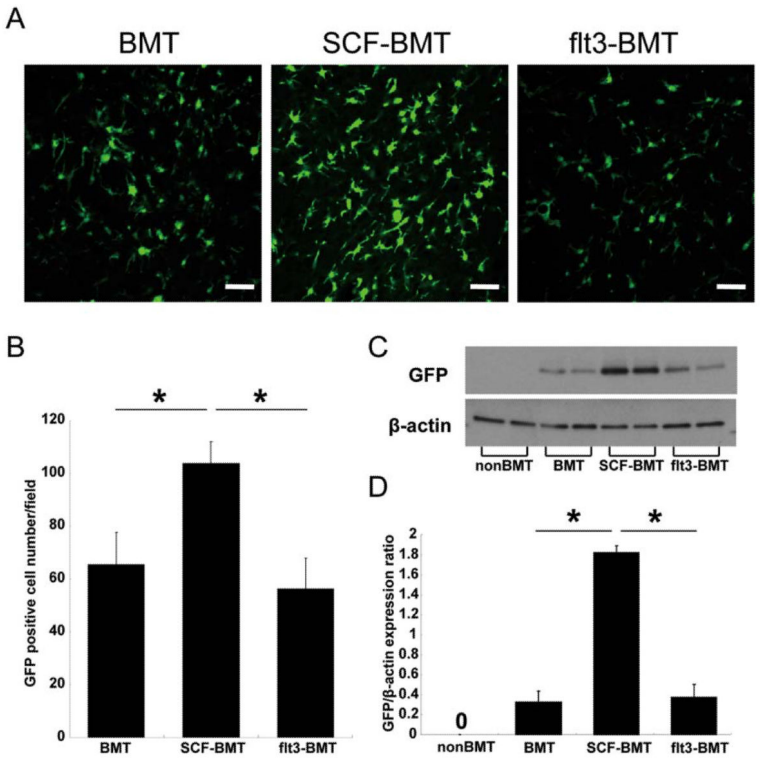

Fig. 4.

Number of GFP-positive cells and level of GFP protein expression in SOD1-tg mice after transplantation with SCF- or flt3-activated BM cells from GFP mice. **A:** GFP-positive cells (green) in spinal cord sections harvested from SOD1 transgenic (SOD1-tg) mice after BM transplantation from GFP-positive BM treated with no growth factor (left, BMT), with SCF (middle, SCF-BMT), or with flt3 (right, flt3-BMT). **B:** Number of GFP-positive cells in spinal cord sections harvested from the three groups (BMT, SCF-BMT, and flt3-BMT; n = 5 in each group). Numbers of GFP-positive cells were standardized by single visual field (400 × 400 μm). Bars show mean ± SD. ★$P < 0.01$. **C:** Immunoblots of GFP protein in spinal cord tissues from SOD1-tg mice in the non-BMT group (non-BMT) and in the BMT groups (BMT, SCF-BMT, and flt3-BMT). The lower row shows immunoblots of β-actin as a standard. **D:** Quantitative analysis of expression of GFP protein in spinal cord tissues from SOD1-tg mice in non-BMT group (non-BMT) and in BMT groups (BMT, SCF-BMT, and flt3-BMT, n = 5 in each group). The ratio of GFP expression was standardized by β-actin expression. Bars indicate the mean ± SD. ★$P < 0.01$. Scale bars = 50 μm.

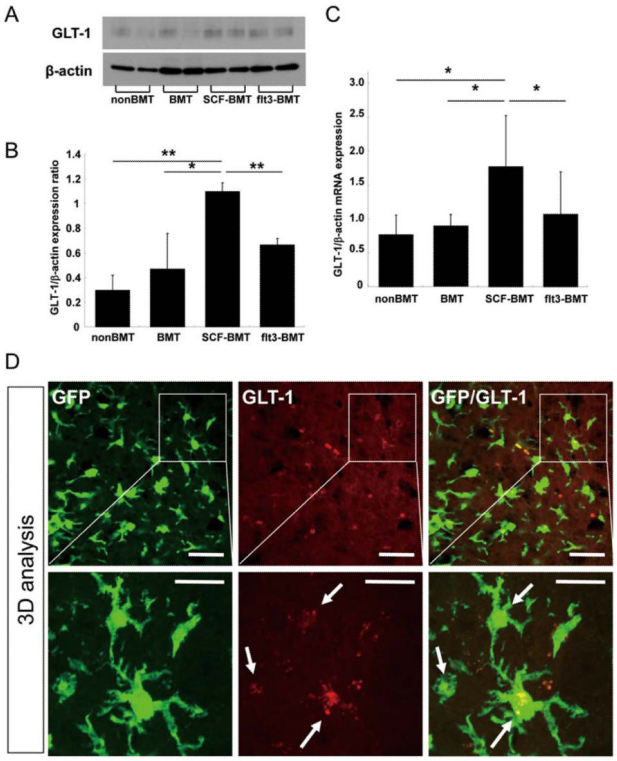

Fig. 5.

GLT-1 protein and mRNA expression in spinal cord tissues from SOD1-tg mice after transplantation with SCF-activated BM cells from GFP mice. **A:** Immunoblots of GLT-1 protein in spinal cord tissues from SOD1 transgenic (SOD1-tg) mice after BM transplantation (BMT) from GFP-positive BM cells not treated with a growth factor (left, BMT) or treated with SCF (middle, SCF-BMT) or flt3 (right, flt3-BMT). The lower lane shows immunoblots of β-actin as a standard. **B:** Quantitative analysis of the expression of GLT-1 protein in spinal cord tissues from SOD1-tg mice in the non-BMT group (non-BMT) and in the BMT groups (BMT, SCF-BMT, and flt3-BMT; n = 5 in each group). The ratio of GLT-1 expression was standardized by β-actin expression. Bars shows mean ± SD. ★P < 0.05, ★★P < 0.01. **C:** Quantitative RT-PCR analysis of GLT-1 mRNA expression in spinal cord tissues from SOD1-tg mice in non-BMT and in BMT groups (BMT, SCF-BMT, and flt3-BMT; n = 5 in each group). The mRNA expression of GLT-1 was standardized by that of β-actin and shown as the ratio against the GLT-1 expression in spinal cord from nontransgenic littermates. Bars show mean ± SD. ★P < 0.05. **D:** Immunohistochemistry with anti-GLT-1 and distribution of GFP-positive BM-derived cells in spinal cords harvested from SOD1-tg mice after BMT with SCF-modified BM cells from GFP transgenic mice. About 20 1-μm-thick specimens (total 20 μm) were merged into one picture for three-dimensional analysis. Boxed areas in the upper row are enlarged below. Arrows indicate GLT-1 positive staining. Scale bars = 50 μm in the top panels; 25 μm in the bottom panels.

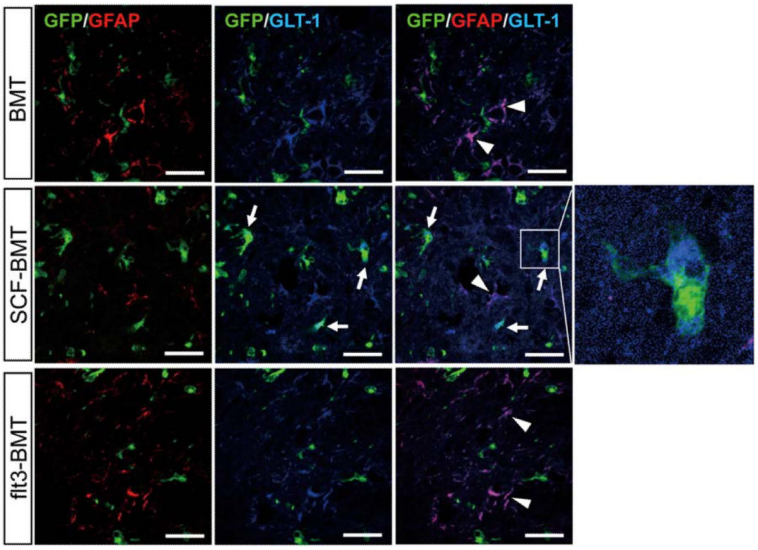

Fig. 6.

Localization of GLT-1 expression in spinal cord tissues in SOD1-tg mice after transplantation with SCF-activated BM cells from GFP mice. Immunohistochemistry with anti-GFAP (red, left and right columns) and anti-GLT-1 (blue, middle and right columns) with GFP signaling were performed in spinal cord sections from SOD1 transgenic (SOD1-tg) mice after transplantation with BM cells treated with no growth factor (upper row, BMT), with SCF (middle row, SCF-BMT), or with flt3 (lower row, flt3-BMT) from GFP transgenic mice. Panels on the left show GFP (green) signals and GFAP (red) stain; middle panels show the GFP (green) signal and the GLT-1 (blue) stain; the third panels shows the merged images [GFP (green), GFAP (red), and GLT-1 (blue)]. The boxed area is enlarged at right. Arrows show cells expressing both GFP and GLT-1. Arrowheads show merged cells with GFAP and GLT-1. Scale bars = 25 μm in the three leftmost columns; 25 μm for the right panel.

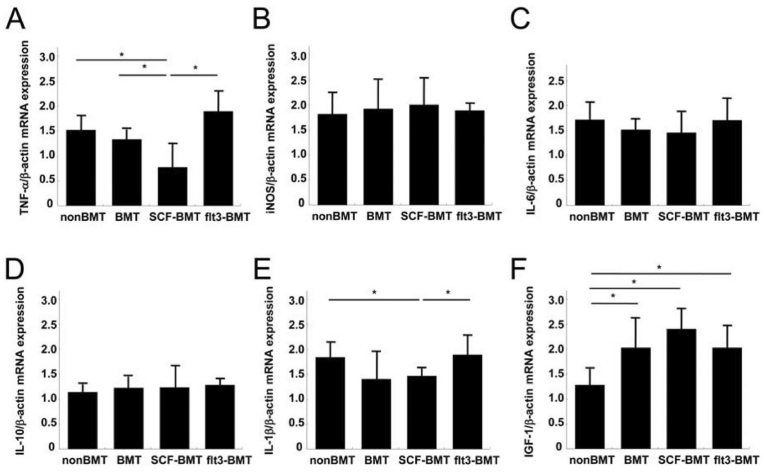

Fig. 7.

Quantitative RT-PCR analysis of mRNA expression of neurotoxic or neuroprotective molecules. **A:** TNF-α. **B:** iNOS. C: IL-6. **D:** IL-10. E: IL-1β. **F:** IGF-1. mRNA expression in spinal cord tissues from SOD1-tg mice in non-BMT and in the BMT groups (BMT, SCF-BMT, and flt3-BMT; n = 5 in each group). The mRNA expression was standardized by the β-actin mRNA expression and shown as the ratio against each mRNA expression in spinal cord from nontransgenic littermates. Bars show mean ± SD. **P* < 0.05.