Granulocyte colony-stimulating factor stimulates neurogenesis via vascular endothelial growth factor with STAT activation

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ARTICLE INFO

Acceptor 6 December 2005
Available online 19 January 2006

Keywords:
Granulocyte colony-stimulating factor (G-CSF)
Neurogenesis
G-CSFR
STAT
VEGF

Abbreviations:
NSC, neural stem cell
DG, dentate gyrus
GCL, granule cell layer
SGL, subgranular layer
SVZ, subventricular zone
RMS, rostral migratory stream
OB, olfactory bulb

ABSTRACT

The adult brain harbors multipotent stem cells, which reside in specialized niches that support self-renewal. Granulocyte colony-stimulating factor (G-CSF) induces bone marrow stem cells proliferation and mobilization from their niche, and activates endothelial cell proliferation, which might help to establish a vascular niche for neural stem cells (NSCs). Here, we show that G-CSF induced receptor-mediated proliferation and differentiation of neural precursors in human NSCs cultures and in adult rat brain in vivo. In human NSCs cultures, G-CSF activated STAT3 and 5, and increased VEGF and its receptor, VEGFR2 (Flk-1) expression, and VEGFR2 tyrosine kinase inhibitor blocked the neurogenesis stimulated by G-CSF. G-CSF also activated endothelial cell proliferation in adult rat brain in vivo. Our results indicate that G-CSF stimulates neurogenesis through reciprocal interaction with VEGF and STAT activation.

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1. Introduction

A variety of adult stem cells were localized in restricted areas of several organs known as “niche” where a subset of tissue cells and extracellular substrates can indefinitely house one or more stem cells and control their self-renewal and progeny production. The adult brain harbors multipotent stem cells, which reside in specialized niches that support self-renewal (Luskin, 1993; Magavi et al., 2000; Shen et al., 2004). Astrocytes, ependymal cells and endothelial cells collaborate to define niches for cell genesis, and reciprocal paracrine interactions between these cells can both permit and regulate neurogenesis or gliogenesis from resident precursor cells. Agents as diverse as epidermal growth factor-2 (FGF-2) (Ray et al., 1993), brain-derived growth factor (BDNF) (Pencea et al., 2001), insulin-like growth factor-1 (IGF-1) (Aberg et al., 2000), stem cell factor (SCF) (Shingo et al., 2001), and vascular endothelial growth factor (VEGF) (Cao et al., 2004; Jin et al., 2002b; Sun et al., 2003) have been implicated as permissive factors for neurogenesis in the adult brain.

Granulocyte colony-stimulating factor (G-CSF), a member of the cytokine family of growth factors, induces BM stem cells (BMSCs) proliferation and mobilization from their niche (Petit et al., 2002). G-CSF exerts its activity via a receptor (G-CSFR) of the hematopoietin receptor superfamily (Cosman, 1993). Binding to G-CSFR recruits cytoplasmic tyrosine kinases of both the Jak and Src kinase families and activates STAT proteins (Shimoda et al., 1997; Tian et al., 1994, 1996). Activated STAT translocates to the nucleus and regulates specific target gene expression, which allows cells to proliferate, differentiate and mobilize or to obtain enough trophic support to survive (Shuai et al., 1993; Tian et al., 1996). By this complex cascade, G-CSF might also regulate the proliferation, migration, or differentiation of the adult neural stem cells (NSCs).

On the other hand, G-CSF directly activates endothelial cell proliferation (Bussolino et al., 1991; Natori et al., 2002). The release of BDNF from endothelial cells can establish a vascular niche that favors the proliferation of neuronal precursors (Louissaint et al., 2002). Besides, STAT3 has been reported to directly regulate VEGF expression and hence angiogenesis in the human pancreatic cancer (Wei et al., 2003). Recently, VEGF has been implicated as a factor that promotes neurogenesis in the adult brain (Jin et al., 2002b; Louissaint et al., 2002), and the kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1; KDR), have been known to be also expressed by nonvascular cells such as neurons or NSCs (Maurer et al., 2003; Sondell et al., 1999). G-CSF-induced STAT activation might operate an autocrine VEGF loop in NSCs, and subsequently enhance the neurogenesis.

Little is known regarding the existence of G-CSFR on human NSCs or possible role of G-CSF in the adult brain. In the present study, we show that it stimulates the proliferation of neuronal precursors in human NSCs cultures and in adult rat brain in vivo. We then elucidate the plausible mechanisms of G-CSF action in the adult brain.

2. Results

2.1. G-CSF stimulates neurogenesis in human NSCs in vitro

HB1.F3 NSCs were bipolar, tripolar or multipolar in morphology and 8–10 μm in size (Fig. 1a). The cells expressed phenotypes specific for NSCs, nestin (100%, Fig. 1b). Most cells expressed markers associated with immature neurons, β-III tubulin (Tuj-1; 68%, Fig. 1c) and doublecortin (Dcx; 65%). The cells were also immunopositive to proliferating cell markers; 60% showed positivity to BrdU and 58% to Ki-67 (Fig. 1d). Within the BrdU+ population, little change in the relative proportion of neurons and astrocytes was observed (Tuj-1: 69%, Dcx: 67%). When these cultures were treated with G-CSF for 72 h, they showed increased incorporation of BrdU into cells (Figs. 1e and f, up to +22% vs. control) that expressed the immature neuronal marker, Tuj1 or Dcx (Fig. 1g; up to +30% vs. control). This effect was concentration-dependent and was associated with an increase in cell number (DAPI-stained nuclei, Fig. 1h; up to +28% vs. control, P = 0.003, n = 4). The concentration-response relationships for stimulation of BrdU labeling and for elevation of cell number by G-CSF were similar, with maximal effects at 50 ng/ml. At higher G-CSF concentrations (~50 ng/ml), the effect tapered off. To document further the mechanisms by which G-CSF induces the cell growth, the cell cycle distribution was determined by measuring DNA content with flow cytometry in cells with or without G-CSF treatment. As shown in Fig. 1i, G-CSF at 50 ng/ml induced a significant reduction in the percentage of cells in G0/G1 (53.7%) and a parallel increase in the percentages of cells in S (31.2%) and G2/M (15.1%) in comparison with untreated control (67.4, 23.6 and 9.0%, respectively) and G-CSF-treated cells at 10 ng/ml (62.2, 26.6 and 11.1%, respectively).

2.2. G-CSF-induced neurogenesis is mediated via G-CSF receptor with subsequent activation of STAT pathway

To identify the receptor mechanism involved in the proliferation of NSCs in vitro by G-CSF, NSCs cultures were immunostained with antibodies against G-CSFR, and total cell lysates from the NSCs treated with or without G-CSF were harvested for the western blot analysis. G-CSFR was expressed in almost all cells (Fig. 2a), and the levels were not altered by G-CSF treatment (Fig. 2b). To explore if STAT activation mediates the effects of G-CSF on cell proliferation, HB1.F3 cell extracts were further analyzed to detect the activated tyrosine-phosphorylated forms of STAT3 (pSTAT3) and STAT5 (pSTAT5). STAT3 and -5 were phosphorylated to some extent even at the steady state. G-CSF treatment increased STAT3/5 phosphorylation (Figs. 2c and d), while total STAT3/5 protein levels were not sensitive to treatment. The optical density analysis (Fig. 2e) showed a 4.2 and 2.7-fold higher expression for pSTAT3 and pSTAT5 respectively, in the G-CSF-treated cells compared with controls.

2.3. G-CSF-induced neurogenesis is potentiated by VEGF secreted from NSCs with upregulation of VEGFR2/Flk-1/KDR

We next investigated whether G-CSF influences VEGF production and VEGF receptor expression in human NSCs. ELISA
analysis revealed that VEGF levels rose from 137.4 ± 13.2 pg/ml at baseline to 163.4 ± 9.6 pg/ml at G-CSF-treated NSCs cultures (50 ng/ml), which was statistically significant (Fig. 3a; \( P = 0.02 \)).

To detect both constitutively expressed and induced receptors, either of which could be involved, western blotting was performed in our HB1.F3 cell cultures. The VEGFR2/Flk-1/KDR receptors were expressed in the untreated control, while VEGFR1/Flt-1 receptors were scarcely expressed. VEGFR1/Flt-1 receptor was highly expressed in the human endothelial cell line ECV.304 which was used as a positive control of our study. The expression of VEGFR2/Flk-1/KDR receptors was upregulated in the G-CSF treated cultures (Fig. 3b). The optical density analysis (Fig. 3c) showed a 2.5-fold higher expression for Flk-1/KDR in the G-CSF-treated cultures compared with controls. To determine if locally secreted VEGF contributes to the neuroproliferative effect of G-CSF, we next examined the effects of VEGF or VEGFR2/Flk-1 receptor tyrosine kinase inhibitor on the neurogenesis stimulated by G-CSF (Fig. 4). The combinatorial treatment of G-CSF (50 ng/ml) and VEGF (50 ng/ml) significantly increased the total number of HB1.F3 cells (Fig. 4a; +19% vs. G-CSF alone, \( P = 0.002 \); +16% vs. VEGF alone, \( P = 0.01 \); +43% vs. control, \( P < 0.001 \)). The combinatorial treatment of G-CSF and VEGF also induced a significant reduction in the percentage of cells in G0/G1 (51.8%) and a parallel increase in the percentages of cells in S (33.2%) and G2/M (15.0%) in comparison with G-CSF or VEGF alone (Fig. 4b). In addition, the VEGFR2/Flk-1 receptor tyrosine kinase inhibitor SU1498 blocked the G-CSF ability to increase the stem cell proliferation (Fig. 4a; −14% vs. G-CSF alone, \( P < 0.001 \), \( n = 4 \)) or to shift the cell cycle to S and G2/M phases from G0/G1 (S: 23.6%, G2/M: 8.4%, G0/G1: 68.0%).

2.4. G-CSF stimulates neurogenesis in SVZ, SGL, and OB in vivo

To evaluate if G-CSF acts as a neurogenic factor in adult brain in vivo, we first examined whether G-CSFR is expressed in proliferative zones in the rat brain. G-CSFR was detectable in the SVZ of adult rats where G-CSF or vehicle is infused (Figs. 5a and b). Numerous scattered neurons and glial cells adjacent to these areas were also stained with the G-CSFR antibody. No difference in the pattern and intensity of G-CSFR immunoreactivity was evident between the two experimental groups. To ascertain whether G-CSF induces in vivo neurogenesis, as demonstrated above in vitro experiments, brain sections from G-CSF- and vehicle-treated rats were processed for double-labeling immunohistochemistry with antibodies against BrdU and cell-type-specific markers (n = 6). Although the distribution of the newly generated cells was similar in both sets of animals,
the BrdU labeling in G-CSF-infused brains was upregulated with subsequent expansion of the SVZ, SGL and rostral migratory stream (RMS) (Fig. 5). From the quantitative analysis, G-CSF increased BrdU-positive cells by 1.5- to 2-fold in the SGL and SVZ, and by 3-fold increase in the olfactory bulb (OB) (Fig. 5; $P < 0.05$, $n = 6$). Cells that incorporated BrdU by 14 days after G-CSF infusion included Tuj-1-expressing immature neurons in the SVZ, SGL and RMS and CB-positive mature neurons in OB (Fig. 6). Quantitative analysis from the OB revealed that G-CSF treatment produced greater amount of cells of neuronal lineage (Fig. 6g; Tuj-1: 1076 ± 92 cells; CB: 1425 ± 123 cells; GFAP: 475 ± 41 cells, $P < 0.05$, $n = 6$) compared to vehicle (Tuj-1: 321 ± 34 cells; CB: 425 ± 45 cells; GFAP: 141 ± 15 cells).

2.5. G-CSF increases vascular density and induces mitotic angiogenesis in the brain

To address the neurogenic mechanisms of G-CSF in vivo, we next asked whether the intraventricular administration of G-CSF also affects the cerebral microvascular profiles and endothelial proliferation. To confirm the identity of endothelial cell division, sections were double-immunolabeled for both BrdU and laminin, a marker of endothelial cells. Figs. 7a–c show that BrdU+ nuclei in the SVZ and OB of G-CSF-infused rats colocalize with immunoreactivity for laminin. Double-labeled cells were common after G-CSF infusion, but extremely rare in vehicle-infused animals, consistent with the normal absence of significant angiogenesis in the SVZ and OB. When counted in enlarged and thin-walled vessels, containing BrdU+ endothelial cells (Fig. 7d), the fraction of BrdU+ endothelial cells in the SVZ and OB was 8.4 ± 2.6% and 3.5 ± 0.7% in the G-CSF treated rats and 2.6 ± 0.7% and 1.7 ± 0.5% in the controls ($P < 0.05$, $n = 6$). We also noticed a dramatic change in blood vessels after G-CSF treatment compared to vehicle (Figs. 7e, f). The mean diameter of the cerebral microvessels was significantly larger in the SVZ and OB of the G-CSF treated rats, as compared with the corresponding value for the control rats (SVZ: 7.6 ± 2.1 μm vs. 4.6 ± 1.9 μm, OB: 6.3 ± 1.7 μm vs. 4.2 ± 0.9 μm; $P < 0.05$, $n = 6$).

3. Discussion

The major finding of this study is that G-CSF, identified originally as a regulator of hematopoietic stem cells, and more recently as an angiogenic factor, can also stimulate neurogenesis, identified by increased BrdU labeling of neuronal precursor cells in vitro and in vivo. G-CSF-induced neurogenesis was associated with phosphorylation of STAT3 and -5, which was probably mediated by interaction with G-CSFR. In addition, G-CSF induced VEGF and VEGFR2/flk-1/KDR expression in NSCs cultures, and the G-CSF effect was blocked by VEGFR2 tyrosine kinase inhibitor.

To identify G-CSF-induced neurogenesis, we employed BrdU labeling in human NSCs cultures. G-CSF increased the NSCs number with dose response and stimulated the incorporation of BrdU into the cells that co-expressed phenotypic markers of neurons. G-CSF might rescue the newly formed cells that would otherwise undergo cell death. However, in our study, simply the prevention of cell death seemed insufficient to account for the immense number of BrdU-positive cells and
the cell cycle shift to S and G2/M phases. The additional finding that G-CSF-induced cell proliferation tapered off at higher concentration than 50 ng/ml, suggested that the neurogenesis by G-CSF might be critically dependent on proper concentration. G-CSF (10-100 ng/ml) significantly induced phosphorylation and activation of Jak2 and STAT3, and to a lesser extent, STAT1 and 5 on bone marrow precursors (Hofer et al., 2005; Li et al., 2005; Liu et al., 2001). The dose of G-CSF (50 ng/ml) that is effective in our in vitro study was similar to that which works on bone marrow precursors.

We also tested the in vivo neurogenic potential by the intraventricular administration of G-CSF. The intraventricular dose of G-CSF was adjusted to the results of the in vitro experiment along with other growth factors (Jin et al., 2002b). The intraventricular route of G-CSF administration could partly exclude a possibility that BrdU-labeled cells in the brain could originate from BM. Neurogenesis persists in restricted adult mammalian forebrain structures, including the SGL of the hippocampal dentate gyrus (Keyt et al., 1996), the SVZ of the lateral ventricle (Luskin, 1993), and the parenchyma of the adult forebrain (Magavi et al., 2000). The neural progenitors, which originate from stem cells located in the SVZ, follow an intricate migration path and reach their final position in the OB (Luskin, 1993). They move tangentially, in clustered chains, along the entire extent of the RMS. Once in the bulb, they turn to move radially out of the RMS into outer layers, when they differentiate into CB-positive GABAergic interneurons (Luskin, 1993). Because the OB is one of the few structures in the adult forebrain in which there is a continuous supply of newborn neurons, we examined the effect of G-CSF on the distribution and phenotype of newly generated cells mainly in SVZ-RMS-OB stream. G-CSF infusion resulted in numerous BrdU-positive cells, not only in the SVZ and SGL, but moreover, in OB. In each region, most of the newly generated cells expressed the neuronal marker, Dcx, Tuj-1 or CB.

To elucidate the mechanism underlying these findings, we examined the G-CSFR and STAT activation. G-CSFR is a single-chain member of the cytokine receptor superfamily, which lacks tyrosine kinase activity (Ihle, 1995). Binding of

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**Fig. 3** ELISA for VEGF and western blot analysis of VEGFR. (a) VEGF concentrations (mean ± SD) were determined in supernatants using ELISA and expressed as pg/ml. VEGF was secreted by HB1.F3 cells to some extent at the steady state, and this was significantly increased by G-CSF (P = 0.02, n = 3). (b) Protein from control cultures or from cultures exposed to G-CSF for 72 h was probed for the principal VEGF receptors with anti-VEGFR2/Flk-1 or anti-VEGFR1/Flt-1 antibodies. (c) VEGFR2/Flk-1 receptors were upregulated by G-CSF treatment, while VEGFR1/Flt-1 receptors are scarcely expressed in the untreated or treated groups. The experiment was repeated three times with similar results. *P < 0.05 compared with untreated control.

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**Fig. 4** The effects of VEGF or VEGFR2/Flk-1 receptor tyrosine kinase inhibitor on the neurogenesis by G-CSF. (a) The total cell number was measured by counting DAPI. (b) Cell cycle was analyzed by flow cytometry using propidium iodide. Maximally effective concentrations (50 ng/ml) of G-CSF and VEGF significantly increased the total cell number (P < 0.01, n = 3), and the cell percentage in the S and G2/M phases compared with the treatment of G-CSF or VEGF alone. G-CSF-stimulated cell proliferation in HB1.F3 cell cultures was inhibited by the VEGFR2/Flk-1 receptor tyrosine kinase inhibitor SU1498 (P < 0.01, n = 3). P < 0.05 compared with G-CSF alone (*) or VEGF alone (#) group, respectively.
G-CSF to its receptor, induces the activation of Jak-1 and Jak-2. In addition to the activation of these Jak kinases, this interaction also leads to the phosphorylation of STAT (Ihle, 1995; Rane and Reddy, 2000). On phosphorylation, STAT translocates to the nucleus and initiates transcription of its target genes, which in turn exhibit pleiotropic effects on many common processes regulating cell fate (Ihle, 1996). The cell cycle is regulated tightly at G1-S and G2-M checkpoints by several protein kinases composed of a cdk subunit and corresponding regulatory cyclin subunit, and cdk inhibitors (Grana and Reddy, 1995). The G1-S checkpoint is controlled by the CIP/KIP and INK4 families of cdk inhibitors, including p21waf1/cip1, p27kip1, and p18ink4c, which bind to G1 cyclin/cdk complexes and inhibit their catalytic activity, thus preventing the transition of cells from G1 to S phase (Sherr and Roberts, 1999). STATs have been shown to control cellular proliferation by regulating p27kip-1 expression (Kaplan et al., 1998). G-CSF was expressed in our cultures and the neuroproliferative zones of the adult rat brain as reported previously (Schneider et al., 2005). While total STAT3 and -5 protein levels were not changed by G-CSF treatment, the phosphorylated STAT3 and -5 protein levels increased, supporting that STAT phosphorylation depends on G-CSF signaling. Cell cycle analysis revealed a significant stimulation of cell cycle progression by G-CSF at the G1-S checkpoint. This result suggests that the G-CSF-induced NSCs proliferation is mediated, at least in part, through cell cycle stimulation at the G1-S checkpoint.

Stimulus-induced G-CSF expression has been reported in human astrocytes (Aloisi et al., 1992; Wesselingh et al., 1990), and Jak-STAT signaling pathways have been implicated in the promotion of astrocytic differentiation and induction of the astrocytic marker, GFAP (Bonni et al., 1997). Thus, G-CSF might be involved in gliogenesis. However, a recent report demonstrated that G-CSF and G-CSFR were expressed predominantly in neurons or neural stem cells in normal or ischemic rat brain rather than astrocytes (Schneider et al., 2005). Moreover, exogenous G-CSF could stimulate neuronal differentiation of adult neural stem cells in vitro rat hippocampal cultures and in vivo rat brain (Schneider et al., 2005). These actions of endogenous or exogenous G-CSF support an involvement of neurons or neuronal precursors in neurogenesis. To clarify the involvement of glial cells in neurogenesis, the receptor–ligand interaction in various cell types and the precise mechanism of cooperation between signaling pathways must be determined.

Many leukemia and cancer cells exhibit constitutive activation of STATs, which was suggested to contribute to...
Fig. 6 – Phenotype of newly generated cells in the adult rat brain. (a–f) Brain sections were immunostained for BrdU (green) and marker of immature (Tuj-1) or mature (Calbindin) neurons (red). Merged images (yellow) showed that a large amount of newly generated cells in the SVZ, RMS and SGL and within the OB expressed a neuronal phenotype. (g) At 14 days after G-CSF infusion, G-CSF treatment produced numerous cells of neuronal lineage in the OB. *P < 0.05 compared with vehicle-treated rats. Scale bars, 50 μm; SVZ: subventricular zone, SGL: subgranular layer, RMS: rostral migratory stream, OB: olfactory bulb.

Fig. 7 – Capillarization and endothelial proliferation in the SVZ and OB. (a–c) Representative photographs show that some BrdU-positive cells (arrows) are laminin immunopositive in the SVZ and OB. (d) Treatment with G-CSF significantly increased laminin+BrdU+ cells in the SVZ and OB. (e, f) The vascular density was more increased in G-CSF-infused rats than vehicle-infused ones. (g) The mean diameter was significantly greater in the G-CSF-infused rats than in controls. *P < 0.05 compared with vehicle-treated rats. n = 6; Scale bars: (a–c), 50 μm (e, f) 100 μm; SVZ: subventricular zone, OB: olfactory bulb.
autonomous growth and tumorigenicity (Moucadel and Constantinescu, 2005). A few studies have identified a neuroprotective effect for erythropoietin, which utilizes STAT5 for transcriptional regulation of anti-apoptotic genes (Reni et al., 2002; Wen et al., 2002). However, the biological effects of Jak/STAT pathway in human NSCs have not been specifically determined. Because we found preferential STAT5 activation on NSCs by G-CSF treatment, we suggest the potential role of STAT5 in neurogenesis. However, post-translational modifications, cofactor interactions and down-regulation might result in cell type specific responsiveness. The fidelity of STAT5-mediated gene regulation needs to be further defined.

In the present study, the upregulation of VEGF and Flk-1/KDR was linked with G-CSF treatment. The recent study has indicated that human pancreatic cancer cells exhibited constitutively activated STAT3, with its activation correlated with the VEGF expression level (Yahata et al., 2003). Constitutively activated STAT3 directly activated the VEGF promoter, and blockade of activated STAT3 via ectopic expression of dominant-negative STAT3 suppressed VEGF expression (Yahata et al., 2003). Similarly, the upregulation of VEGF on NSCs by G-CSF might depend on G-CSFR-Jak-STAT3 pathway. VEGF is not only an angiogenic factor but a directly-acting neurogenic factor, and it regulates the proliferation and differentiation on neural precursors independent of its effects on endothelial cells (Bonni et al., 1997; Yahata et al., 2003). The recent evidence suggested an internal autocrine loop mechanism, by which VEGF could regulate the proliferation and differentiation of NSCs when co-expressed with its receptors (Maurer et al., 2003). VEGF is regulated by the Flt-1 and Flk-1/KDR receptors, which are part of the tyrosine kinase family. Flk-1/KDR is involved in cell proliferation, while Flt-1 is thought to mainly regulate the formation of cellular architecture (Keyt et al., 1996). In our study, G-CSF induced VEGF secretion from NSCs. NSCs expressed Flk-1/KDR receptors, but not Flt-1, and Flk-1/KDR could be upregulated by G-CSF. VEGFR2 receptor tyrosine kinase inhibitor blocked the effects of G-CSF on cell proliferation. These observations suggest that G-CSF-induced neurogenesis needs VEGF signaling. The combination of G-CSF (50 ng/ml) and VEGF (50 ng/ml) significantly increased the total number of NSCs (+43% vs. control) compared to G-CSF alone (+24% vs. control) or VEGF alone (+27% vs. control). The effect (+43%) of combinational treatment of G-CSF and VEGF was lower than that (+51%) of G-CSF alone plus VEGF alone. This non-additive finding indicated that they might act through a shared signal transduction pathway (Bartoli et al., 2000; Yahata et al., 2003). It is very important to test whether G-CSF effect is blocked and VEGF level is changed by inhibiting Jak/STAT pathway. Further experiments are needed to dissect out the signal transduction mechanisms implicated, e.g. inhibitor peptides, genetic controls including dominant negative STAT or KDR, and ideally genetic knockdown of the growth factor and receptor.

We have found that significant angiogenesis takes place within the SVZ and OB after G-CSF treatment. G-CSF stimulates the proliferation of cerebral endothelial cells directly (Bussolino et al., 1991). On the other hand, our findings that VEGF and Flk-1/KDR were upregulated on NSCs themselves by G-CSF suggest an additional mechanism that NSCs may contribute to neovascularization by direct angiogenic effects. In normal brain, the neurogenesis in the adult brain occurs in intimate association with angiogenesis in neurogenic zones of the hippocampus (Palmer et al., 2000), and songbird HVC (Louissaint et al., 2002). Furthermore, factors released by endothelial cells in vitro trigger NSCs proliferation (Shen et al., 2004). G-CSF might act upon the local microvascular bed to provide a permissive environment for neuronal recruitment into the brain.

Our results indicate that G-CSF stimulates neurogenesis through reciprocal interaction with VEGF and STAT activation. The coordinate induction of angiogenesis and neurogenesis by a common factor can have critical value, by efficiently assisting the cell replacement therapy. Taken together with the recent evidences that G-CSF can rescue dying neurons (Schäbitz et al., 2003; Schneider et al., 2005), G-CSF might potentially serve to promote brain recovery and repair.

4. Experimental procedure

4.1. Human neural stem cell cultures

All experimental procedures were approved by the Care of Experimental Animals Committee of Seoul National University Hospital and by institutional review board for the human cell use. Primary dissociated cell cultures were prepared from embryonic human brains of 15 weeks gestation as described previously in detail (Cho et al., 2002; Chu et al., 2003, 2004; Flax et al., 1998; Jeong et al., 2003; Ourednik et al., 2002). Maintaining NSCs in a proliferative state in culture did not subvert their ability to respond to normal developmental cues in vivo following transplantation (Chu et al., 2003, 2004; Jeong et al., 2003). The cells were initially grown as a polyclonal population first in serum-supplemented and then in serum-free medium containing basic FGF and/or EGF. The cultures were retrovirally transduced with the v-myc oncogene and subsequently cloned. One of the isolated clones, HB1.F3, was expanded for the present study. The cells were grown on poly-L-lysine-coated culture dishes or flasks as single cells or large clusters, which could be subcultured and passaged weekly over a period of 6 months. The cultures were maintained in a serum-free medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) containing insulin (10 μg/ml), transferrin (10 μg/ml), sodium selenite (30 μM), hydrocortisone (50 μg/ml), and triiodothyronine (0.3 μM). The human recombinant G-CSF ([Kirin Pharmaceuticals, Tokyo, Japan] was added to cultures for 72 h at various concentrations.

4.2. Quantification of viable cells

Cell viability was assayed by MTT absorbance and by counting cells on photomicrographs of 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). For MTT assays, cultures were incubated with a stock solution of MTT (5 mg/ml in PBS, pH 7.4, Sigma) at 37 °C for 4 h at a final concentration of 1 mg/ml, and absorbance at 570 nm was measured in solubilized cells on the ELISA reader. For cell counting, the number of intact DAPI-stained nuclei in five 200×
microscope fields per well (at the 3-, 6-, 9-, and 12-o’clock positions and in the center) was recorded. In both cases, results were expressed as a percentage of values obtained in control cultures not treated with G-CSF.

4.3. BrdU labeling in vitro

Cells were plated on 12 mm round Aclar plastic coverslips previously coated with 10 μg/ml polylysine and housed in 35 mm dishes. BrdU (10 μg/ml; Sigma) was added for 72 h to the cultures exposed to G-CSF or not. BrdU-positive cells in culture were counted in five fields per well (center and at 3, 6, 9, and 12 o’clock). Cells containing densely red-stained nuclei were considered BrdU positive, and profiles with smaller or dot-like labeling were considered necrotic cells and were excluded in the analysis. Results were expressed as a percentage of the number of intact DAPI-stained nuclei obtained in the same fields.

4.4. Immunohistochemistry

Cell cultures or brain sections were processed for immunocytochemistry as described previously (Cho et al., 2002; Chu et al., 2003, 2004; Jeong et al., 2003; Jung et al., 2004). The specimens were fixed in 4% paraformaldehyde in PBS (pH 7.5) for 40 min, washed with PBS, incubated for 2 h with blocking buffer (2% horse serum/1% BSA/0.1% Triton X-100 in PBS, pH 7.5), incubated overnight at 4 °C with primary antibodies, and then incubated for 1 h at room temperature with secondary antibodies. Primary antibodies were as follows: monoclonal anti-BrdU (1:300, Pharmingen); sheep polyclonal anti-BrdU antibodies (1:300, BioDesign); anti-Ki67 antibody (1:200, Boeringer-Ingelheim; ABC kit); anti-G-CSFR (1:500, BD Biosciences); anti-Tuj1 (1:200, Chemicon); anti-Dcx (1:100, Santa Cruz Biotechnology); anti-NeuN (1:200, Chemicon); anti-GFAP (1:1000, Sigma); anti-Calbindin (1:200, Sigma); anti-laminin (1:100, Sigma). Biotinylated goat anti-mouse IgG (ABC, sigma) and FITC-conjugated anti-sheep IgG (1:100, Biodesign) or Cy3-conjugated anti-mouse IgG antibodies (1:300, Jackson ImmunoResearch) were used for the secondary antibodies. DAPI (Vector Laboratories) was used to counterstain nuclei in some experiments. The colocalization was analyzed using a laser scanning confocal microscopy with a Bio-Rad MRC 1024 (argon and krypton). The fluorescence signals were detected at excitation/emission wavelengths of 550/570 nm (Cy3, red), 488/522 (FITC, green), and 400/490 (DAPI, blue). Biotin signals were detected with 3,3′-diaminobenzidine (DAB; brown) and Vector VIP (purple).

4.5. Cell cycle analysis

The cell cycle of HB1.F3 cells treated with or without G-CSF was analyzed by FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). At 72 h after treatment, the cells were harvested by centrifugation, counted, and prepared for cell cycle analysis. Cells (5 × 10^4) were suspended in 200 μl of hypotonic propidium iodide (PI) solution (0.1% w/v sodium citrate, 0.1% w/v Triton X-100, 0.05 mg/ml PI) and were incubated in the dark at 4 °C for 24 h before analysis. The cell histogram FL-2 was divided into 3 regions according to cell cycle phase: G0/G1, S, or G2/M. Doublets were eliminated by gating on a peak/area plot of PI fluorescence. The data were analyzed with WinCycle software (Phoenix Flow Systems, San Diego).

4.6. Western blot analysis

We examined the potential signaling molecules involved in neurogenesis only at maximally effective concentration. Western blotting was used to determine the expression of G-CSFR on HB1.F3 cells and subsequent activation of STAT proteins following G-CSF treatment. We also tested what changes are made by G-CSF treatment in the expression of VEGFR1/Flt-1 or VEGFR2/Flk-1 on HB1.F3 cells. The cells cultured on the 100 mm plate were washed with 4 °C PBS and collected. They were homogenized in a lysis buffer (100 mmol/l NaCl, 10 mmol/l Tris (pH 7.5), 1 mmol/l EDTA) to which the protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mmol/ml aprotinin) were freshly added. The protein concentrations were determined using the Bradford method (Bio-Rad, Richmond, CA). The protein extracts (30 μg) were separated by 7–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. They were blocked in 5% non-fat dry milk in TBS (0.15 M NaCl, 25 mM Tris–HCl, 25 mM Tris) for 2 h and then incubated overnight at 4 °C with anti-G-CSFR (1:500, BD Biosciences), anti- VEGF1/Flt-1 (1:500, Santa Cruz Biotechnology), anti-VEGFR-2/Flk-1 (1:500, Santa Cruz Biotechnology), anti-STAT3 (1:1000, Cell Signaling Technology), anti-p-STAT3 (1:1000, Cell Signaling Technology) or anti-p-STAT5 (1:1000, Cell Signaling Technology) antibodies. After washing three times in TBST (TBS + 0.5% Tween-20), the membrane was incubated with the secondary antibody conjugated to horseradish peroxidase at a 1:5000 dilution for 1 h at room temperature. The blots were developed with enhanced chemiluminescence (Fierce), digitally scanned (GS-700, Bio-Rad) and analyzed (Molecular analyst®, Bio-Rad). The relative optical densities were obtained by comparing the measured values with the mean value of the normal control cultures.

4.7. VEGF enzyme-linked immunosorbent assay (ELISA)

Concentrations of VEGF in the culture supernatants treated with or without G-CSF were measured using the Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN), which is a quantitative immunometric sandwich enzyme immunoassay. A curve of the absorbance of VEGF versus its concentration in the standard wells was plotted. By comparing the absorbance of the samples with the standard curve, we determined the VEGF concentration in the unknown samples.

4.8. Co-treatment with G-CSF and VEGF or VEGFR tyrosine kinase inhibitor

To investigate the potential role of VEGF on G-CSF action, the cultures treated with G-CSF were also placed with the recombinant VEGF-165 (50 ng/ml, Calbiochem) or VEGFR tyrosine kinase inhibitor, SU1498 (10 μM, Calbiochem) throughout the time course of the experiments (Jin et al., 2002b). Viable cell number was quantified by MTT assay and by counting cells on DAPI staining and cell cycle was analyzed in each experiment.

4.9. Implantation and use of osmotic minipumps containing G-CSF or saline alone

We investigated whether the intraventricular infusion of G-CSF increases the proliferation of new neurons in the adult rodents.
Twelve Sprague-Dawley male rats \((n = 6\) for G-CSF or vehicle alone-treated groups) weighing 220 to 240 g (Genomics) were used. The osmotic mini-pumps (Alzet, model 2002, Palo Alto, CA) contained 0.9% saline or recombinant G-CSF at the concentrations of 10 μg/ml in saline. The pumps were designed to deliver the contents at 0.5 μl/h for 7 days. The G-CSF concentration would, therefore, deliver a total of 1 μg G-CSF over the week. It had been tested for safety in a pilot project, and no significant side effects were observed. The filled pumps were incubated overnight in sterile saline at 37 ℃ before use. Surgery was performed under the anesthesia with an intraperitoneal injection of 1% ketamine (30 mg/kg) and xylazine hydrochloride (4 mg/kg) as reported previously (Jung et al., 2004). The cannula was placed in the left lateral ventricle 4.0 mm deep to the pial surface and +0.0 mm anteroposterior relative to bregma and 1.8 mm lateral to the midline. All cannula (30 gauge, 4 mm length; Plastics One, Roanoke, VA) were cemented (Cranioplastic cement; Plastics One) to the skull, anchored with machine screws (Plastics One), and connected to the osmotic mini-pump by medical grade vinyl tubing (Biolab Inc., Decatur, GA). All pumps were implanted subcutaneously in the nape of the neck, and the cannula was lowered after the pumps began pumping.

4.10. Quantification of BrdU-positive cells in vivo

For in vivo studies, BrdU (100 mg/kg) was administered intraperitoneally once daily for 7 consecutive days from the day of pump insertion, as reported previously. The rats were sacrificed 7 days after G-CSF or vehicle withdrawal (day 14) to permit newly generated cells to integrate in the host brain \((n = 6\) for each group). BrdU-positive cells were characterized by double-labeling with neuronal or glial cell markers. BrdU-positive cells were counted in five to seven 30 μm coronal sections per animal, spaced 200 μm apart, by two investigators blinded for group allocation. We selected for comparisons corresponding coronal sections, determined using the rat atlas (Paxinos et al., 1997) in both the G-CSF-infused and saline-infused brains. The counts were made, using a 40× objective, by placing an optical grid (field size, 250 μm × 250 μm). The density of BrdU-labeled cells, expressed as cells per cubic millimeter, was determined in the SVZ, SGL, RMS and OB. The number of double-labeled BrdU+Tuj1+, BrdU+CB1+, and BrdU+GFAP+ cells in the SVZ, SGL, RMS, and OB was counted with a conventional or confocal microscope.

4.11. Capillary parameters and endothelial proliferation

The vasculature was analyzed using a rabbit anti-laminin IgG (1:100, Sigma). Sections were permeabilized by 0.1% saponin for 15 min, blocked with 10% goat serum for 1 hr, and exposed to anti-laminin IgG overnight at 4 °C. After washing, the primary anti-laminin antibody was detected with Alexa Fluor 566-conjugated goat anti-rabbit IgG (1:400, Molecular Probes). In each sampled olfactory bulb, every laminin-positive profile was traced and recorded manually into image analysis system (Image-Pro Plus™). A profile was defined as the net surface area of laminin immunolabeling. Capillary counts were derived from these profiles, and no correction was made for tangential cuts. This was based upon an assumption of randomly distributed capillary orientations in coronal sections. Each traced outline was then analyzed for its diameter, and the average values per olfactory bulb were determined. Endothelial cell division was detected by double staining for BrdU and laminin. BrdU-immunostained sections were digitized using a ×40 objective (Olympus BX51) via the image analysis system (Image-Pro Plus™). The numbers of total endothelial cells and the numbers of BrdU+ endothelial cells in 20 enlarged and thin-walled vessels in the SVZ and OB were counted from each rat \((n = 6\) per group), from which the proportions of BrdU-positive endothelial cells were calculated.

4.12. Statistical analysis

All data in this study are presented as mean ± standard deviations. Data were analyzed by repeated measures of analysis of variance, and unpaired Student’s t test, if they were normally distributed (Kolmogorov–Smirnov test, \(P > 0.05\)). Otherwise, we used Mann–Whitney U test and specified the test used. \(P\) values of less than .05 were considered to indicate a statistically significant difference.

Acknowledgments

This study was supported by the 21st Century Frontier Research Fund of the Ministry of Science and Technology (SC3060), Republic of Korea. G-CSF was kindly provided by Kirin Pharmaceuticals. Conflict of interest: None to disclose.

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