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Systemic Application of Granulocyte-Colony Stimulating Factor and Stem Cell Factor Exacerbates Excitotoxic Brain Injury in Newborn Mice

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ABSTRACT: Granulocyte-colony stimulating factor (G-CSF) has been shown to reduce brain lesion size and mortality in adult mice after hypoxic-ischemic injury. Another hematopoietic growth factor, stem cell factor (SCF), has been shown to be up-regulated in the brains of adult rodents following brain damage, where it stimulates postlesional neurogenesis. Injection of the excitotoxic agent ibotenate into the brain of newborn mice produces a brain lesion characterized by neuronal death and white matter cysts, which is similar to periventricular leucomalacia. The aim of the present study was to investigate whether administration of SCF and G-CSF is neuroprotective against ibotenate lesions in neonatal mice. Contrary to our expectations, cortical and white matter brain lesions induced by ibotenate were enhanced following the administration of 50 μg/kg SCF or 200 μg/kg G-CSF. Dose-response studies indicated that G-CSF could increase grey matter lesions even at lower dosages (22 and 66 μg/kg). Administration of SCF and G-CSF in combination also increased cortical and white matter lesions, to 133 $\pm$ 8% and 187 $\pm$ 12%. In the undamaged brain, G-CSF or G-CSF+SCF treatment had no effect on apoptosis in the grey or white matter; however, these treatments significantly increased apoptosis in the damaged brain. Our data clearly demonstrate that G-CSF and SCF are not neuroprotective and result in deleterious enhancement of excitotoxic brain damage in newborn mice. We conclude that G-CSF and SCF should be used cautiously in newborn infants with brain lesions; if they are used, long term neurologic and neurodevelopmental follow-up is warranted. **(*Pediatr Res* 59: 549–553, 2006)**

A significant proportion of preterm infants develop periventricular white matter damage, including periventricular leukomalacia (PVL). Preventative and/or protective measures are difficult to apply, since the injury is thought to occur before or around birth (1) and the early markers of white matter damage remain elusive. Therefore, it appears necessary to develop therapies that enhance the repair of the damaged brain. Until recently, it was thought that the CNS cannot repair or regenerate itself (2). But the dogma that neuronal replacement is not possible after brain injury has been challenged by recent data indicating that neuronal progenitor cells exist in the adult mammalian brain, not only in the subventricular area but also in other brain areas (3). Further, it has been shown that neurogenesis occurs in response to cerebral injury, including excitotoxic damage (4), seizures (5) and oxidative stress-induced apoptosis (6). One candidate factor that might stimulate increased neurogenesis is stem cell factor (SCF). SCF expression has recently been shown to be increased in neurons following brain damage and was speculated to mediate the migration of neuronal progenitor cells to areas of acute brain injury (7,8). Exogenous administration of recombinant SCF has also been found to increase neurogenesis (9).

Another approach that has been pursued to reduce/repair brain damage involves bone marrow stem cells (BMSC), which are mobilized by SCF and granulocyte-colony stimulating factor (G-CSF) and have been shown to cross the blood brain barrier (migrating even into the undamaged brain), where they differentiate into neural and glial cells (10). Since G-CSF is already undergoing clinical trial for use in newborns, this therapeutic approach may represent a feasible means for reducing already acquired brain damage in preterm infants.

In the present study, we hypothesized that SCF alone or in combination with G-CSF would be neuroprotective in newborns. To test this hypothesis, we used a well characterized mouse model of neonatal excitotoxic brain damage, which mimics many key features of human PVL (11). This hypothesis was tested independently at two different laboratory sites.

MATERIALS AND METHODS

Materials. Ibotenic acid was obtained from Biotrend (Germany), anti-cleaved caspase 3 antibody from Cell Signaling (New England Biolabs, Germany), PBS (PBS) from Invitrogen (Austria), biotinylated goat anti-rabbit IgG and Vectastain ABC from Vector Laboratories, Burlingame, CA. Fluorochrome-labeled antibodies directed toward murine CD45, CD34 and CD117 were obtained from Pharmingen, San Diego, CA. Anti-CD45, anti-F4/F8 from Caltag Laboratories, Burlingame, CA, and anti-Gr1 from eBioscience,

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Abbreviations: BMSC, bone marrow stem cells; **G-CSF,** granulocyte-colony stimulating factor; **IC,** intracranial; **IP,** intraperitoneal; **PVL,** periventricular leukomalacia; **SCF,** stem cell factor

Amersham Pharmacia, Piscataway, NJ. Flow-Count™ fluorospheres were obtained from Beckman Coulter, Fullerton, CA. All other substances, including murine G-CSF, murine SCF and MK801, were obtained from Sigma Chemical Co. (Germany).

Excitotoxic brain damage. We used the animal model of excitotoxic brain damage described by Marret *et al* (12). Five-day-old CD1 mice were obtained from the animal facility at the University of Innsbruck or from Elevage Janvier (Le Genest-St-Isle, France). They were housed under standard conditions in a conventional animal facility. Adequate measures were taken to minimise pain or discomfort, complying with the European Community guidelines for the use of experimental animals. All pups investigated were anesthetized with isoflurane and kept warm. Intra-cerebral injections were performed with a 26-gauge needle on a 50 μL Hamilton syringe mounted on a calibrated microdispenser. The needle was inserted stereotactically 2 mm under the external surface of the scalp in the frontoparietal area of the right hemisphere, 2 mm from the midline in the lateral- medial plane and 3 mm from the junction between the sagittal and lambdoid sutures in the rostro-caudal plane. Two 1 μL boluses were injected 2 mm and 1 mm under the surface 20 s apart. The needle was left in place for 20 s after the second bolus. After injection, the animals were returned to their dams. Histopathology confirmed that the tip of the needle always reached the periventricular white matter.

Experimental groups. Pups investigated received either no insult or an intra-cerebral injection of ibotenate (10 μg) on postnatal day 5 to cause an excitotoxic brain lesion. Experiments were performed at two laboratories, one in Innsbruck, Austria and one in Paris, France.

In the first set of experiments (carried out in Innsbruck), mouse pups were divided into five experimental groups: one single intraperitoneal (IP) injection of (1) PBS (PBS) or (2) G-CSF (200 μg/kg) + SCF (50 μg/kg), given one hour after ibotenate injection and analyzed 24 h after setting the lesion; four IP injections of (3) PBS or (4) G-CSF (200 μg/kg/d) + SCF (50 μg/kg/d), given once per day for four days starting 24 h following ibotenate injection; and (5) one single IP injection of G-CSF (200 μg/kg) + SCF (50 μg/kg), given one hour after ibotenate injection. Groups (3) to (5) were analyzed 120 h after setting the lesion.

In the second set of experiments (carried out in Paris), pups were divided into three experimental groups: (1) one single IP injection of SCF (50 μg/kg), applied one hour after ibotenate injection; and four IP injections of (2) PBS or (3) SCF (50 μg/kg/d), given once a day for four days starting 24 h after ibotenate injection. All groups were analyzed 120 h after ibotenate injection.

Dose-response studies (experimental set three) with various concentrations of G-CSF were perfomed. Animals were randomized into four groups after the excitotoxic insult and treated with an IP injection one hour after setting the lesion as follows: (1) PBS (2) 22 μg/kg G-CSF (3) 66 μg/kg G-CSF and (4) 200 μg/kg of G-CSF. A fifth group of animals were treated with the NMDA receptor antagonist MK801 (1 mg/kg, IP), 30 min before intra-cerebral ibotenate injection and a subsequent single dosage of G-CSF (200 μg/kg). All five groups were analyzed on day 10 of life (5 d following treatment). To assess the effect of G-CSF treatment on the number of granulocytes, a second set of animals (treatment groups 1-4 as described above) were analyzed at day 6 of life.

The absolute dose of SCF and G-CSF was adapted daily to the current body weight of the pups. In both centers, the animals were injected with ibotenate before randomization into their treatment group; consequently, pups from at least 5–10 separate litters randomly contributed the groups, without account for the female:male ratio.

Lesion size determination. Mouse pups were euthanized by decapitation either 24 or 120 h after the excitotoxic injury. Brains were immediately fixed in 4% formaldehyde for 72 h. Following paraffin embedding, 15 μm thick coronal sections were sliced. Every third section was stained with cresyl violet and the lesion size was determined as described previously (11). Briefly, neocortical and white matter lesions can be defined by the maximal length of three orthogonal axes: the lateral–medial axis (in a coronal plane), the radial axis (also in a coronal plane, from the pial surface to the lateral ventricle) and the fronto-occipital axis (in a sagittal plane). In previous studies, we have shown an excellent correlation between the maximum size of the different diameters the excitotoxic lesions. Based on these observations, we serially sectioned the entire brain in the coronal plane. This permitted an accurate and reproducible determination of the maximum sagittal fronto-occipital diameter (which is equal to the number of sections where the lesion was present multiplied by 15 μm) and this was used as an index of the volume of the lesion. In a subset of experimental groups (third experimental set: ibotenate alone, ibotenate + G-CSF 200 μg/kg, $n = 8$), brains were serially cut in the sagittal plane, permitting the determination of the maximal medio-lateral diameter of the lesion; this diameter was compared with the fronto-occipital diameter. At both centers, two observers independently determined lesion size, each blinded to the treatment groups being analyzed. Data are presented as mean length of the lesion in rostro-caudal axis ± SEM. The numbers of pups used in each experiment are depicted in the bars of each figure.

Immunohistochemistry for cleaved caspase-3. Sections adjacent to the lesion were reacted with anti-cleaved caspase-3 antibody (Cell Signalling, New England Biolabs, Germany). In detail: 10 μm of 4% formaldehyde-fixed and paraffin-embedded mouse brain sections were deparaffinized in xylene, passed through graded alcohols, and endogenous peroxidase activity was quenched by incubation in 2% hydrogen peroxide in methanol for 30 min. After incubation in PBS, heat induced antigen retrieval was performed using 10 mM citrate buffer, pH 6.0, for 15 min in a microwave oven. After non-specific blocking with 10%FCS in PBS, sections were incubated overnight at 4°C with polyclonal anti-cleaved caspase-3 antibody (1:500 dilution; Cell Signaling). After rinsing with PBS, sections were incubated for 45 min at 25°C with biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories, Burlingame, CA, USA). After washing with PBS, sections were incubated for 45 min at 25°C with a streptavidin-biotin-complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). Visualisation was performed using diaminobenzidine as the chromogen. Following multiple rinses in distilled water, sections were dehydrated, cleared in xylene and coverslipped. Labelled cells in the neocortical layers and underlying white matter at the level of ibotenate-induced lesions at the right and left hemisphere were counted by observers blinded to the groups. Ten non-adjacent fields from five brains were studied in each group.

Flow cytometric analysis of leucocytes, granulocytes and hematopoietic stem cells. For detection of stem cells, 100 μL of whole blood was stained with fluorochrome-labeled antibodies directed toward murine CD45, CD34 and CD117 (all from Pharmingen, San Diego, CA). For quantification of granulocyte numbers, blood samples were stained with anti-CD45, anti-F4/F8 (Caltag Laboratories, Burlingame, CA), and anti-Gr1 (eBioscience, Amersham Pharmacia, Piscataway, NJ). In addition, a defined volume of Flow-Count™ fluorospheres (Beckman Coulter, Fullerton, CA) was added to all samples for determination of absolute cell numbers. Following incubation for 15 min, samples were treated twice with erythrocyte lysing solution (Becton Dickinson, San Diego, CA) to eliminate the presence of red blood cells. Cells were then re-suspended in PBS and immediately analyzed by flow cytometry (FC500, Beckman Coulter). At least 10,000 cells were recorded in each sample.

Statistics. Quantitative data are expressed as the mean length of lesion size along the sagittal fronto-occipital axis ± SEM for each treatment group. Results were statistically compared with a *t* test or an ANOVA followed by *t* tests with Dunnett's correction for multiple comparisons of means (Sigma Chemical Co. Stat for Windows Version 2.03). Statistical differences were considered to be significant when $P < 0.05$.

RESULTS

G-CSF + SCF exacerbates excitotoxic brain damage; effect on hematopoietic stem cells, leucocytes and granulocytes. Control pups injected intra-cerebrally with ibotenate and IP with PBS developed typical cortical lesions, with large neuronal loss in all layers and typical white matter cysts, as described previously (12). In animals treated with a single dose of G-CSF + SCF, lesion sizes were not significantly different from controls in white and grey matter after 24 h (372 ± 33 μM in controls *versus* 372 ± 35 μM in treated pups for white matter; 539 ± 55 μM *versus* 551 ± 39 μM for grey matter, $n = 14$). The effect of single and repetitive application of G-CSF and SCF on excitotoxic brain damage after 120 h is displayed in Fig. 1. Both single and repetitive application of G-CSF + SCF significantly increased the white matter lesion size after 120 h ($p < 0.05$). Grey matter lesion size was significantly increased only by the repetitive application ($p < 0.05$).

We also analyzed the effect of G-CSF + SCF treatment on the number of leukocytes, granulocytes and BMSC. As shown in Figure 4*A–B*, one dose of G-CSF + SCF had no significant effect on the amount of BMSC, compared with PBS controls. In contrast, this single injection treatment led to a pronounced increase in leukocytes and granulocytes ($n = 8$, $p < 0.05$

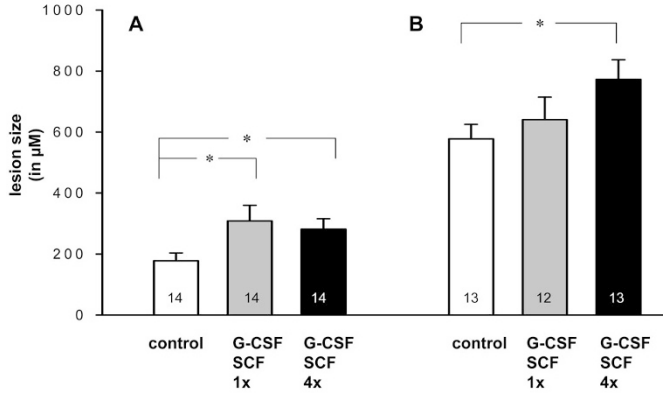

Figure 1. G-CSF and SCF after excitotoxic brain damage. Quantitative effects of G-CSF (200 μg/kg) + SCF (50 μg/kg) IP treatment on ibotenate-induced (A) white matter and (B) grey matter lesions. Lesions were produced on P5 and analyzed on P10. Bars represent mean length of the lesions in the fronto-occipital axis ± SEM. Asterisks indicate statistically significant differences from PBS-treated controls ($p < 0.05$, ANOVA followed by t tests with Dunnet's correction). Numbers in the bars represent the number of animals used in each experimental group (n = 12–14).

versus control). The repeated IP treatment with G-CSF + SCF significantly increased the amount of BMSC (25 ± 5 n/μL in PBS treated controls *versus* 76 ± 20 n/μL, $p < 0.05$) leukocytes and granulocytes ($p < 0.05$ *versus* PBS treated controls).

SCF alone increases lesion size. In animals treated with a single dose of SCF (50 μg/kg), given one hour after the excitotoxic insult or with four daily injections starting 24 h after the insult, significantly increased the lesion size of both white and grey matter lesions, when compared with PBS treated controls (Fig. 2, n = 12, $p < 0.05$).

G-CSF alone increases lesion size. G-CSF (200 μg/kg) applied one hour after the excitotoxic insult increased brain damage in grey and white matter in the fronto-occipital axis (n = 14, $p < 0.05$, Fig. 3) and the medio-lateral axis (n = 8, $p < 0.05$). There was no significant difference in the mean length of the lesion between the fronto-occipital and the medio-lateral axis (1130 μM ± 43 μM (n = 8) in the

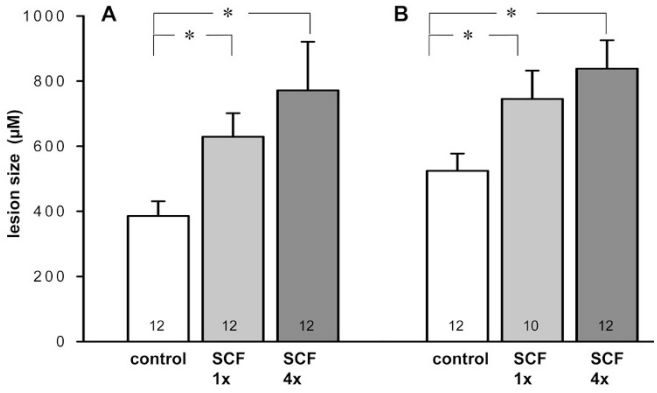

Figure 2. SCF alone after excitotoxic brain damage. Quantitative effect of SCF (50 μg/kg) IP treatment on ibotenate-induced brain lesions. Lesions were produced on P5 and analyzed on P10. Bars represent mean length of the lesions in the fronto-occipital axis ± SEM. Asterisks indicate statistically significant differences from PBS-treated controls ($p < 0.05$, ANOVA followed by t tests with Dunnet's correction). Numbers in the bars represent the number of animals used in each experimental group (n = 10-12).

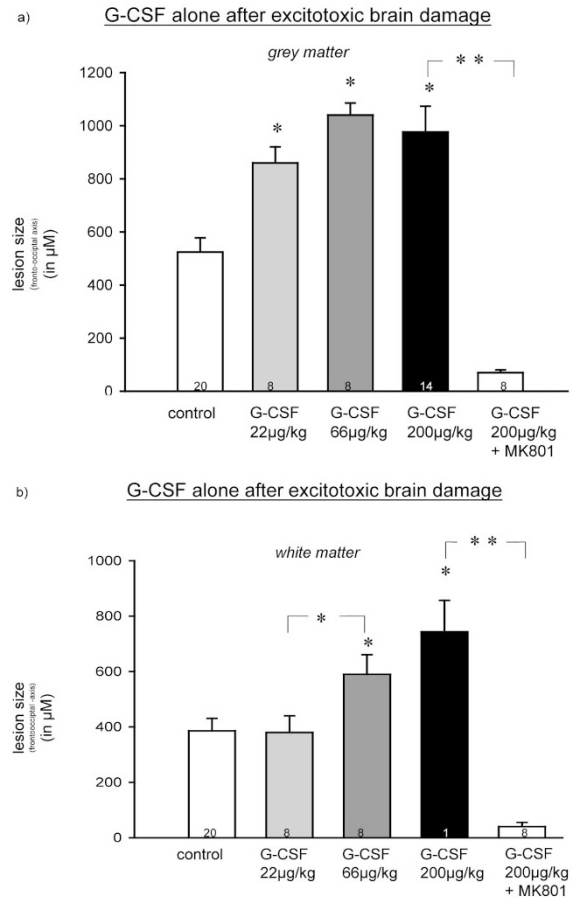

Figure 3. G-CSF alone after excitotoxic brain damage. Quantitative effects of G-CSF (22 μg/kg, 66 μg/kg, 200 μg/kg) and MK801 IP treatment on ibotenate-induced grey matter (*A*) and white matter (*B*) lesions. Lesions were produced on P5 and analyzed on P10. Bars represent mean length of the lesions in the fronto-occipital axis ± SEM. Asterisks indicate statistically significant differences from PBS-treated controls ($p < 0.05$, ANOVA followed by t tests with Dunnet's correction). Numbers in the bars represent the number of animals used in each experimental group (n = 8–20).

medio-lateral axis *versus* 976 ± 96 μM (n = 14) in the fronto-occipital axis in grey matter, 680 ± 42 μM (n = 8) in the medio-lateral axis *versus* 743 ± 113 *versus* the fronto-occipital axis in white matter (n = 14)). As displayed in Fig. 3, G-CSF in dosages of 22 and 66 μg/kg also significantly increased the length of the lesions in grey matter in the fronto-occiptal axis (n = 8, $^*p < 0.05$), whereas the lesion size in white matter was not significantly affected (Fig. 3*B*) n = 8, $p < 0.05$ *versus* control).

Pre-treatment with MK801 completely blocked the excitotoxic lesions in white and grey matter (Fig. 3*A–B*, n = 8, $^{**}p < 0.01$ *versus* G-CSF 200 μg/kg treated animals).

The various concentrations of G-CSF differentially affected the number of granulocytes in blood (Fig. 4*C*). Whereas 22 μg/kg G-CSF had no effect, 66 and 200 μg/kg G-CSF significantly increased the number of granulocytes compared with the PBS-treated control animals (n = 6, $p < 0.05$)

G-CSF and SCF increase apoptosis in damaged but not in undamaged brain. G-CSF (200 μg/kg) treatment and G-CSF + SCF in combination did not affect the number of caspase-3 positive cells in non-damaged brains (cortex and white matter

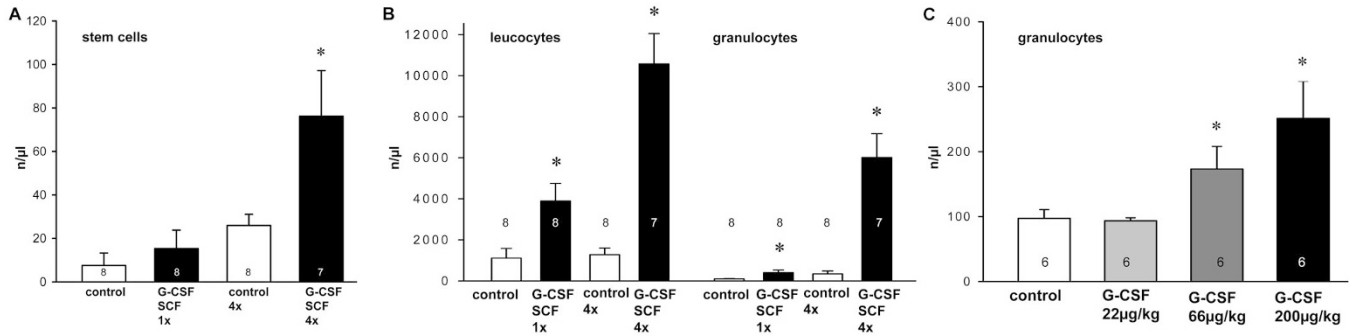

Figure 4. G-CSF and SCF after excitotoxic brain damage. *A,B*: Quantitative effects of G-CSF (200 µg/kg) + SCF (50 µg/kg) single and repetitive IP treatment on *A*) hematopoietic stem cells and *B*) on leucocytes and granulocytes. *C*) Quantitative effect of G-CSF (22 µg/kg, 66 µg/kg, 200 µg/kg) on the amount granulocytes. Data are given as number of cells/µL ± SEM at P6 and P10. Asterisks indicate statistically significant differences from PBS-treated controls ($p < 0.05$, ANOVA followed by *t* tests with Dunnet's correction). Numbers in the bars represent the number of animals used in each experimental group ($n = 6–8$).

at the level of hippocampus) compared with PBS treated animals (data not shown, $n = 5$). However, as shown in Fig. 5, in animals with excitotoxic insult, G-CSF (200 µg/kg in a single dosage) significantly elevated the number of apoptotic cells in the cortex and white matter. This effect was not restricted to the area of the insult (right side) but was also seen in the undamaged left hemisphere. The same apoptosis-increasing effect was seen when animals were treated with the combination of G-CSF and SCF ($n = 5$, $p < 0.05$ *versus* control treated animals).

DISCUSSION

The hypothesis of this investigation was that treatment of newborn mice with SCF or G-CSF would reduce an artificially-induced excitotoxic brain injury that mimics periventricular leucomalacia lesions in human infants. Previous reports strongly suggested that a neuroprotective effect of SCF and G-CSF would occur (9,13). However, this hypothesis must be rejected based on our results, which clearly demonstrate in several sets of experiments conducted in separate laboratories that SCF and G-CSF, both alone or in combination, exacerbate neonatal excitotoxic brain lesions. These results, which were obtained from a newborn mouse model, are in disagreement with reports from adult rodent models.

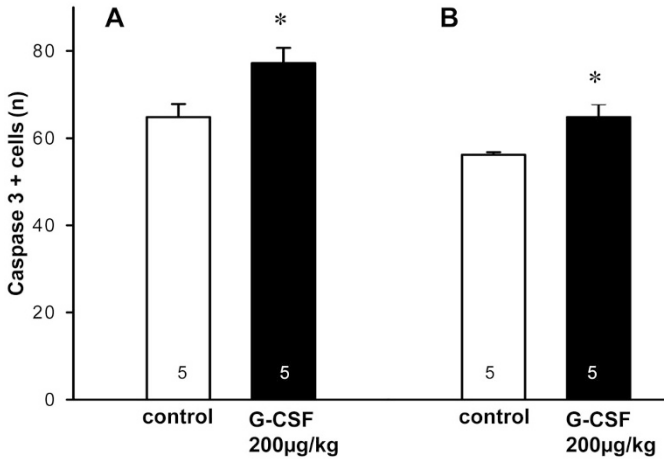

Figure 5. G-CSF alone after excitotoxic brain damage. Quantitative effects of G-CSF (200 µg/kg) IP treatment on ibotenate-induced apoptosis in grey matter and the underlying white matter. Data are presented as number of positive stained cells ± SEM in the (*A*) right (damaged) and the (*B*) left (undamaged) hemisphere at P10. Asterisks indicate statistically significant differences from PBS-treated controls ($p < 0.05$, Student's *t* test). Numbers in the bars represent the number of animals used in each experimental group ($n = 5$).

In an animal model of myocardial infarction, repetitive IP injection of G-CSF and SCF increased the endogenous release of undifferentiated BMSC (14), which has been reported to significantly reduce the size of the infarct and improve the myocardial function (15,16). In an analogous study, a single dosage of G-CSF administered after cerebral hypoxia/ischemia led to an impressive reduction of mortality and of the infarcted brain volume (13). In an animal model of intracerebral hemorrhage in adult rats, G-CSF treatment reduced brain edema, peri-hematomal cell death, and inflammation (17). These discrepancies in our results may be due to the differences in the nature of the lesion created, as well as the differences in animal's age and thus brain development and maturation.

The deleterious effect of G-CSF and SCF to the neonatal mouse brain was only observed in the presence of the excitotoxic brain damage. This led us to speculate that the enhancing effect on lesion size is due to an augmentation of the inflammatory response initiated by the excitotoxic brain damage. This could be due to: 1) the unspecific stimulation of the systemic immune system, consistent with the observed increase in granulocytes, or 2) a direct effect within the brain on neuronal and glial cells. We have previously shown that microglial activation plays a critical role in the pathophysiology of neonatal excitotoxic brain lesions (11). Since SCF can also modulate microglial activity, apart from stimulating neurogenesis (18), this effect could be responsible for a different result of SCF treatment in the present model. Since G-CSF is already used in neonatology clinical trials, we focused on G-CSF and performed a dose-response study. Our results led us to speculate that the exacerbation of brain damage by G-CSF alone might be due to the fact that G-CSF increases the availability of neutrophils (19), which therefore enhances the inflammatory response in the brains of newborns. On the other hand, the fact that the enhanced lesion size in grey matter

appeared to be independent of the dosage of G-CSF is suggestive for a direct effect of G-CSF within the brain. A possible mechanism could be a modification of microglial cell function since it has been recently shown that G-CSF increases the number of microglial cells in cultures treated with G-CSF (20).

To our knowledge, the present study includes the first analysis of the effect of G-CSF and SCF on the release of HSC in newborn mice. It should be noted that a single dosage did not have a significant effect on the number HSC in blood. This contradicts the conclusion of Six *et al* (13), where the mobilization of bone marrow contributed to the positive effect of a single dosage of G-CSF on experimental cerebral ischemia. However, our results are in accordance with a report from Bodine *et al* (21), which showed that the increased release of bone marrow stem cells after the application of G-CSF and SCF occurs with a significant delay. According to Bodine *et al* (21), the elevation in circulating stem cells was only observed at day 4, after daily IP injections of SCF and G-CSF in adult mice.

Our results do not necessarily exclude a potential beneficial role of bone marrow stem cells, which could stimulate the production of substances critical for neurorepair. It is possible that G-SCF and SCF could exert a beneficial effect when applied after the inflammatory response initiated by the excitotoxic brain damage has subsided.

Our results emphasize the fact that treatment results obtained in adult animal models cannot necessarily be transferred to the developing organism, including newborn infants. Further, our results could have important clinical implications: G-CSF is already being used in clinical trials to treat or prevent neonatal infections (22). In these clinical trials, acute adverse or toxic effects of G-CSF have not been demonstrated (23) but, to our knowledge, the analysis of neurological outcome has not been evaluated. The underlying mechanism(s) of G-CSF and SCF toxicity on excitotoxic neonatal brain lesions remains to be determined and this study should be repeated using a different animal model of perinatal brain damage. Our results, obtained independently from two laboratories, clearly demonstrate that G-CSF and SCF, given separately or in combination, have no neuroprotective effect, but rather a deleterious impact on neonatal excitotoxic brain damage in mice.

We conclude that cautious use of the hematopoietic growth factors SCF and especially G-CSF in newborn infants with brain lesions is indicated and, if used at all, long-term neurologic and neurodevelopmental follow-up is warranted.

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