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Long-term evaluation of granulocyte-colony stimulating factor on hypoxic-ischemic brain damage in infant rats

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Abstract

Purpose—Hypoxia-ischemia (HI), as a major cause of fetal brain damage, has long-lasting neurological implications. Therefore, therapeutic interventions that attenuate the neuropathological outcome of HI while also improving the neuro-functional outcome are of paramount clinical importance. The aim of this study was to investigate the long-term functional and protective actions of granulocyte-colony stimulating factor (G-CSF) treatment in an experimental model of cerebral HI.

Methods—Postnatal day-7 Sprague-Dawley rats were subjected to HI surgery, which entailed ligation of the right common carotid artery followed by 2 h of hypoxia (8% O₂). Treatment consisted of subcutaneous injection of G-CSF at 1 h after hypoxia followed by an additional one injection per day for 5 days (6 total injections) or for 10 days (11 total injections). Animals were euthanized 5 weeks post-insult for extensive evaluation of neurological deficits and assessment of brain, spleen, heart, and liver damage.

Results—G-CSF treatment promoted somatic growth and prevented brain atrophy and under-development of the heart. Moreover, reflexes, limb placing, muscle strength, motor coordination, short-term memory, and exploratory behavior were all significantly improved by both G-CSF dosing regimens.

Conclusions—Long-term neuroprotection afforded by G-CSF in both morphological and functional parameters after a hypoxic-ischemic event in the neonate provides a rationale for exploring clinical translation.

Keywords

G-CSF; Neurobehavior; Neonatal; Neuroprotection; Stroke; Hypoxia-ischemia

Introduction

Neonatal hypoxia-ischemia (HI) is a major cause of long-term neurological disturbances, such as behavioral alterations and motor deficits including cerebral palsy, mental retardation, and epilepsy [1, 2]. In spite of advancements in obstetric and neonatal intensive care, HI brain damage with severe neurological sequelae remains an important clinical problem [3]. Therefore, the efficacy of potential neuroprotective treatments on long-term functional brain recovery is of significant translational importance.

Granulocyte-colony stimulating factor (G-CSF), a neurotropic factor involved in proliferation, differentiation, and functional integration of neural cells [4], is a neuroprotective agent in a wide spectrum of experimental models of neurological disease [5–11]. Protection ranges from reductions in infarct size during the acute phase to attenuation of long-term functional neurological deficits [12, 13]. Promotion of neurogenesis and promotion of angiogenesis are processes by which G-CSF exerts beneficial effects on adult post-stroke recovery [14–16]; however, due to the vast differences in the pathophysiology of immature and adult brains, data gathered from adult studies do not necessarily imply synonymous outcomes in neonatal medicine. Therefore, the effects of G-CSF treatment on cerebral infarct volume at 2 weeks [17] and cerebral atrophy at 3 weeks [6] after HI have previously been explored. No study to date, however, has examined whether these G-CSF-induced morphological benefits translate into improvements to sensorimotor deficits and memory 5 weeks after neonatal HI and whether there is an additive benefit against long-term brain and systemic organ atrophy provided with additional administration of G-CSF.

Accordingly, we hypothesized that G-CSF has lasting neuroprotective actions by improving both morphological and behavioral endpoints after HI injury in neonatal rats. We used two different dosing regimens to clarify the neurotrophic capabilities of G-CSF in terms of structural preservation and improved functional outcome.

Materials and methods

Animal groups and surgical procedure

This study was in accordance with the National Institutes of Health guidelines for the treatment of animals and was approved by the Institutional Animal Care and Use Committee at Loma Linda University. Postnatal day-7 Sprague-Dawley rats were randomly assigned to the following groups: sham, HI (vehicle), HI + G-CSF daily for 5 days [G-CSF(5d)], or HI + G-CSF daily for 10 days [G-CSF(10d)]. HI-groups were anesthetized with 3% isoflurane and had the right common carotid artery permanently ligated followed by 1.5 h of recovery. Afterwards, pups were placed in a glass jar (submerged in a water bath maintained at 37°C) perfused with 8% O₂/92% N₂ for 2 h. Sham rats had the common carotid artery exposed, but not ligated. All rats were killed 5 weeks after HI surgery under general anesthesia [ketamine (80 mg/kg)/xylazine (10 mg/kg)] by decapitation.

Treatment method

Some pups were treated subcutaneously with G-CSF (Amgen, Thousand Oaks, CA) at 50 µg/kg dosage diluted in saline. Treatment was injected at 1 h after hypoxia followed by an

additional one injection per day for 5 days (6 total injections) or for 10 days (11 total injections). Vehicle pups received subcutaneous injections of saline following the same regimen.

Evaluation of brain damage and systemic organ weight

The HI animal model results in brain damage exclusive to the ipsilateral side [18, 19], commonly assessed by hemispheric brain weight loss, which is highly correlated to histological loss of brain tissue [20, 21]. Brain tissue [sham: $n = 5$; vehicle: $n = 13$; G-CSF(5d): $n = 12$; G-CSF(10d): $n = 13$] was removed, and the hemispheres were separated by a midline incision and weighed on a high-precision balance (sensitivity ± 0.001 g). Data are expressed as the ratio of ipsilateral (right) to contralateral (left) hemispheric weights. The heart, spleen, and liver were also isolated and weighed. Data for systemic organs are expressed as the ratio of organ weight to body weight.

Assessment of neurobehavioral deficits

The behavior of the rats was blindly evaluated using eight sensorimotor (postural reflex, back pressure, lateral pressure, proprioceptive limb placing, lateral placement, forelimb placement, foot-fault, and rotarod) tests [2, 20]; and a test (T-maze) to ascertain short-term or working memory, as well as complex cortical function [22, 23]. Methodology was as previously described for the first six sensorimotor tests [2] and scored accordingly: 0 for immediate and correct placement, 1 for delayed and/or incomplete placement, 2 for no placement. Scores corresponded to raw values: 0 score = 100; 1 score = 50; 2 score = 0. In the foot-fault test, the rat was placed on a horizontal grid floor (36×13 in, square size 3×3 cm, wire diameter 0.4 cm) for a duration of 2 min. A foot-fault was noted when a paw fell through an opening in the grid floor. In the rotarod test, rats were placed on a rotating treadmill (diameter 14 cm) initially at rest (stationary) for a maximum of 1 min. In the second round of testing, the treadmill was set in motion at a constant speed of 5 rotations per min (rpm) for a maximum of 1 min. Finally in the third round of testing, the treadmill was set in motion at an accelerated speed of 5–40 rpm for a maximum of 2 min. Each animal had two trials/round of testing. The time spent by the animal on the rotarod during each round was noted. In the T-maze test, rats were placed at the base of the T-maze (stem 40×10 cm, arm 46×10 cm) and allowed to explore until an arm of the maze was chosen. Each animal was given 10 trials, and the sequence of right and left arm choices is expressed as the percent of spontaneous alternation.

Data analysis

Data are expressed as mean $\pm$ SEM. Using a commercially available software (Sigma Stat 3.5, Aspire Software, Ashburn, VA), one-way ANOVA and Tukey test were implemented to determine significance in differences between groups. Kruskal–Wallis ANOVA followed by Dunn's test was used for neurobehavioral analysis. Significance was accepted at $p < 0.05$.

Results

G-CSF promotes physical development

HI-induced somatic growth retardation starting from 1 day after insult is a common finding in animal experiments [3, 24]. Representative pictures demonstrate the significant differences in physical development between rats injected with vehicle or G-CSF, at the completion of the 5 day dosing regimen or the 10 day dosing regimen (Fig. 1a). Vehicle rats gained significantly less weight than sham after 1 week following HI (7.73 ± 0.42 vs. 13.38 ± 0.42 g; Fig. 1b); an effect attenuated by both G-CSF treatment regimens (5 days: 10.71 ± 0.53 g; 10 days: 10.49 ± 0.62 g). After 3 weeks following HI, G-CSF(5d) continued to

improve weight gain as compared to vehicle (66.54 ± 1.85 vs. 58.87 ± 1.89 g). Vehicle pups however did catch up in weight since the amount of weight gained over the entirety of the study was not significantly less than that of sham rats (125.01 ± 3.30 vs. 136.12 ± 7.40 g). On the other hand, rats treated with 5 days of G-CSF (143.39 ± 3.58 g) gained significantly more weight over 5 weeks as compared to vehicle rats, but did not differ from those treated for 10 days with G-CSF (136.09 ± 4.60 g).

G-CSF maintains brain and systemic organ weight

Neonatal encephalopathy involves multiple organs and not just the brain [25, 26]; therefore, effects of treatment interventions should be explored across multiple organ systems [27]. HI injury resulted in significant brain atrophy of the lesioned hemisphere ($19.60 \pm 3.09\%$); remarkably, treatment with G-CSF (5 days: $8.70 \pm 2.18\%$; 10 days: $9.70 \pm 2.39\%$) resulted in less damage to the brain tissue 5 weeks post-insult (Fig. 2a, b). Although vehicle rats appeared to have a smaller heart compared to sham, statistical significance was not reached. When compared to G-CSF(5d)-treated rats, vehicle rats had a 16.11% reduction in heart-to-body ratio. Although G-CSF(5d) treatment appeared to additionally improve spleen and liver weights, no statistical differences were detected between groups.

G-CSF ameliorates HI-induced functional deficits

In all behavior tests, vehicle rats performed significantly worse than sham. This showed the consistency with which severe damage was induced across all tested brain regions. Numbers in parenthesis represent mean raw score. In the postural reflex test, the contralateral forelimb of the vehicle rats was completely flexed (16.67 ± 6.29); while treatment with G-CSF(5d) and G-CSF(10d) significantly improved this deficit (5 days: 72.22 ± 8.78 ; 10 days: 66.67 ± 8.33 ; Fig. 3). In the remaining five placement tests, the vehicle rats averaged a raw score ranging from 20.00 ± 6.55 to 43.33 ± 4.54 ; this meant vehicle rats were consistently unresponsive in these tasks. The G-CSF(10d) significantly improved these deficits across tasks, and G-CSF(5d) did so in all but the proprioceptive limb placing test. Rats subjected to HI displayed significantly reduced sensorimotor coordination as assessed using the foot-fault test compared to sham (34.20 ± 2.27 vs. 12.4 ± 1.57). Both G-CSF treatment for 5 days (21.11 ± 1.76) and 10 days (17.56 ± 0.99) significantly attenuated the HI-induced deficits. Vehicle rats also displayed a significant reduction in muscle strength and motor coordination compared to sham (32.57 ± 3.45 vs. 50.24 ± 2.54), as assessed by the rotarod test. Treatment with G-CSF (5 days: 50.49 ± 2.94 ; 10 days: 58.02 ± 1.04) improved the latency to fall period when the treadmill was stationary. When set at constant velocity, G-CSF (5 days: 54.39 ± 2.38 ; 10 days: 59.49 ± 0.51) significantly improved the HI-induced (40.57 ± 4.14) deficits. During acceleration of the treadmill, animals from both G-CSF regimens (5 days: 56.81 ± 4.10 ; 10 days: 65.83 ± 4.11) again performed significantly better than vehicle (32.44 ± 2.46). In the T-maze, vehicle rats demonstrated a significant reduction in exploratory behavior and short-term memory as compared to sham (33.33 ± 3.07 vs. $73.33 \pm 2.72\%$). These deficits were significantly improved by both G-CSF regimens (5 days: $56.79 \pm 4.32\%$; 10 days: $61.73 \pm 1.95\%$); however, there was a difference in performance between G-CSF(5d) rats and sham.

Discussion

This study demonstrates the long-term efficacy of G-CSF administration on behavioral and neuropathological recovery in an established rat model of neonatal HI injury. To our knowledge, our data are the first to demonstrate the effect of multiple treatments of G-CSF on HI-induced sensorimotor and memory impairment. The importance of the present findings may be highlighted by (1) the elucidation of a beneficial treatment regimen with long-term brain and system organ protection, and (2) the significant improvement in

neurological function across a battery of tests, which renders these results important for the treatment of neonatal encephalopathy in the clinical setting.

Experimental stroke studies have found treatment with G-CSF to be well tolerated without major side effects [28]; side effects may include mild to moderate bone and/or musculoskeletal pain, anemia, thrombocytopenia, and injection site reactions [29]. Nevertheless, the safety profile of G-CSF appears to be fairly innocuous, even after years of administration to patients with severe neutropenia [29]. In experimental neonatal HI, a single dose of G-CSF immediately after hypoxia is neuroprotective 2 weeks following insult [17], while multiple doses confer morphological benefits up to 3 weeks [6]. Based on these findings, as well as evidence that neuronal damage likely develops over a period of time [30] and that neuroreparative processes may need stimulation after 24 h when the inflammatory responses have declined [31], we chose to administer multiple doses of G-CSF over 5 days or 10 days following HI. The efficacy of the treatment regimen was determined by its ability to improve physical development, protect brain tissue, and improve long-term functional outcome.

Fetal growth retardation is an HI-related outcome and can be used as an indicator of general well-being [17]. From the body weight data, it is evident that G-CSF treatment significantly improves physical development during the critical period following brain injury. Whilst both treatment protocols initially promoted weight gain after HI, the rats treated with G-CSF(5d) gained significantly more weight at the end of 5 weeks compared to their vehicle-administered counterpart. Moreover, previous studies have shown that low body weight is accompanied by decreased heart weight [32]—an effect we found to be counteracted by G-CSF(5d). An explanation has been that slow-growing pups have a significantly lower number of cardiomyocytes, as well as qualitative changes in the subcellular structures [32]. Other systemic organs affected by fetal growth retardation are the spleen and liver, possibly due to oxygen deprivation and inadequate macro- and micronutrients during fetal life resulting from the preferential blood flow to vital organs such as the brain and heart [33]. However, our results show that these acute effects do not have long-term implications and that the neonatal rat compensates as there were no differences found between groups when comparing spleen and liver weights.

Although no significant differences were found between groups, the G-CSF(5d) treated animals appeared to have a larger body, heart, spleen, and liver weights compared to those in the sham group. This finding may spark concern about unintentional organ hypertrophy upon prolonged treatment with growth factors. In fact, spleen enlargements have been reported during repetitive G-CSF administration—a likely result of G-CSF-induced extramedullary hematopoiesis [34]. However, the observed trend toward organ hypertrophy in the group treated for 5 days may be entirely incidental since more prolonged treatment (i.e., 10 days) did not demonstrate the same trend. Aside from promoting weight gain, G-CSF treatment also protected brain integrity in the neonate. Both G-CSF(5d) and G-CSF(10d) prevented the long-term loss of brain tissue. The anti-apoptotic [6], anti-inflammatory [35], and excitoprotective [36] properties of G-CSF may be responsible for the long-term attenuation in brain damage; however the exact mechanism is yet unclear.

Preservation of structural integrity by G-CSF treatment resulted in improved motor performance. Brain regions, such as the sensorimotor cortex and hippocampus, are critical for the maintenance of sensorimotor function and are adversely affected by HI insult [37]. Accordingly, damage to these vulnerable brain regions severely affected functional performance of vehicle rats. Conversely, G-CSF-induced morphological protection manifested into muscle strength, motor coordination, reflexes, limb placing, short-term memory, and exploratory behavior similar to that of control animals. The recovery processes

that are activated and/or amplified by G-CSF and thereby exert beneficial effects on post-HI recovery are yet to be elucidated.

G-CSF is an attractive candidate as a therapeutic modality for the human neonate and may be expanded as treatment to other clinical fields that share common pathophysiological features, such as neonatal stroke, global cerebral ischemia, and neonatal hemorrhagic brain injury. In the clinical setting, G-CSF has been administered to neutropenic neonates with sepsis once daily for 3–5 days. Based on this, and the positive results we obtained from 5 day treatment with G-CSF, the most relevant time period of treatment in the human might be a 5 day dosing regimen. Our experimental study (i.e., use of a clinically relevant animal model, multiple treatment regimens, and neurobehavioral assessment) provides a foundation for exploring clinical translation. However, we anticipate the need for a dose response study, as well as a more comprehensive evaluation of safety [38] before moving G-CSF to the clinical setting as treatment against neonatal hypoxic-ischemic brain damage.

Conclusions

Overall, the findings from the present study provide new insight into the therapeutic repertoire of G-CSF. Specifically, treatment with G-CSF attenuates long-term brain damage and spares the functional integrity responsible for behavior after neonatal hypoxia-ischemia.

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Fig. 1.

Effect of G-CSF on body weight. Postnatal day-7 rats were subjected to hypoxia-ischemia (HI) induced by permanent ligation of the right common carotid artery followed by 2 h hypoxia (8% O₂). Subcutaneous treatment with granulocyte-colony stimulating factor (G-CSF) began 1 h following hypoxia and continued daily for 5 days [*G-CSF(5d)*] or 10 days [*G-CSF(10d)*]. *Vehicle* animals underwent HI without G-CSF treatment. Sham animals were used as control. **a** Marked differences in physical development at the completion of 5 days of G-CSF treatment (*G-5d*) or 10 days of G-CSF treatment (*G-10d*), as compared to vehicle. **b** Vehicle rats gained significantly less weight at 1 and 3 weeks post-HI but appeared to catch up to weight of sham rats after 5 weeks. Both treatment regimens improved weight gain at the 1 week time point, and the G-CSF(5d) rats had a significantly greater average weight gain than vehicle rats at all tested intervals. Data represent mean $\pm$ SEM; * $p < 0.05$ versus sham, # $p < 0.05$ versus vehicle

Fig. 2. Effect of G-CSF on organ weight. **a** Significant loss of right-to-left hemispheric (*RH:LH*) weight ratio is evident in vehicle rats and improved by G-CSF(5d) and G-CSF(10d). G-CSF(5d) significantly improved heart-to-body weight ratio as compared to vehicle. **b** Representative pictures of organs. Data represent mean $\pm$ SEM; * $p < 0.05$ versus sham, # $p < 0.05$ versus vehicle

Fig. 3.

Functional outcome at 5 weeks after HI. Sensorimotor test scores: 100 for immediate and correct placement; 50 for delayed and/or incomplete placement; 0 for no placement. Vehicle rats did significantly worse than sham in all tests: postural reflex, back pressure, lateral pressure, proprioceptive limb placing (*P. limb placing*), lateral placement, and forelimb placement. G-CSF(5d) improved deficits in all but the *P. limb placing* test, while G-CSF(10d) corrected all tested deficits. Vehicle rats had a greater number of foot faults during the 2 min testing interval, and both treatment regimens attenuated these deficits. In the rotarod test, vehicle rats performed significantly worse under all testing conditions: stationary, constant velocity (5 rpm) and accelerating (5–40 rpm). In the T-maze test, vehicle and G-CSF(5d) rats alternated significantly less between the two arms of the maze as compared to sham; however, G-CSF(5d) and G-CSF(10d) performed significantly better at this task than vehicle rats. Data represent mean $\pm$ SEM; * $p < 0.05$ versus sham, # $p < 0.05$ versus vehicle