Published in final edited form as:

Neurochem Res. 2014 November ; 39(11): 2085–2092. doi:10.1007/s11064-014-1399-7.

Granulocyte colony stimulating factor reduces brain injury in a cardiopulmonary bypass-circulatory arrest model of ischemia in a newborn piglet

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Abstract

Background—Ischemic brain injury continues to be of major concern in patients undergoing cardiopulmonary bypass (CPB) surgery for congenital heart disease. Striatum and hippocampus are particularly vulnerable to injury during these processes. Our hypothesis is that the neuronal injury resulting from CPB and the associated circulatory arrest can be at least partly ameliorated by pre-treatment with granulocyte colony stimulating factor (G-CSF).

Material and Methods—Fourteen male newborn piglets were assigned to three groups: deep hypothermic circulatory arrest (DHCA), DHCA with G-CSF, and sham-operated. The first two groups were placed on CPB, cooled to 18°C, subjected to 60 min of DHCA, re-warmed and recovered for 8-9 hrs. At the end of experiment, the brains were perfused, fixed and cut into 10 µm transverse sections. Apoptotic cells were visualized by *in-situ* DNA fragmentation assay (TUNEL), with the density of injured cells expressed as a mean number ± SD per mm².

Results—The number of injured cells in the striatum and CA1 and CA3 regions of the hippocampus increased significantly following DHCA. In the striatum, the increase was from 0.46±0.37 to 3.67±1.57 (p=0.002); in the CA1, from 0.11±0.19 to 5.16±1.57 (p=0.001), and in the CA3, from 0.28±0.25 to 2.98±1.82 (p=0.040). Injection of G-CSF prior to bypass significantly reduced the number of injured cells in the striatum and CA1 region, by 51% and 37%, respectively. In the CA3 region, injured cell density did not differ between the G-CSF and control group.

Conclusion—In a model of hypoxic brain insult associated with CPB, G-CSF significantly reduces neuronal injury in brain regions important for cognitive functions, suggesting it can significantly improve neurological outcomes from procedures requiring DHCA.

Keywords

apoptosis; cardiac surgery; developing brain; hippocampus; striatum; TUNEL

Introduction

Granulocyte-colony stimulating factor (G-CSF), a member of the cytokine family of growth factors, is a glycoprotein broadly present within the central nervous system (CNS). It has been used extensively in the treatment of chemotherapy-induced neutropenia, as well as in bone marrow reconstitution and stem cell mobilization [1, 2]. It has been shown to promote angiogenesis [3], to be neuroprotective in the model of Parkinson's disease [4], and to improve memory in animal models of Alzheimer's disease [5]. Intravenously administered G-CSF is capable of permeating the intact blood–brain barrier and is safe for use in humans [6, 7]. It has been studied in a variety of brain injury models with very promising results. Exogenous administration of G-CSF has neuroprotective effects in a variety of stroke models [7-9] where neurogenesis is induced near the damaged area, leading to neurological and functional recovery [7,10,11]. Neuroprotective effects of G-CSF may occur by a variety of mechanisms, with the most significant being its anti-inflammatory and anti-apoptotic properties [7, 12-14]. We have previously determined, in a model of ischemic brain injury (cardiopulmonary bypass (CPB) and deep hypothermic circulatory arrest (DHCA), that G-CSF decreases pro-inflammatory and pro-apoptotic activities while simultaneously augmenting anti-inflammatory and anti-apoptotic activities [15,16].

The goal of the present study was to determine whether the observed early effects of GCSF on apoptotic and inflammatory signaling pathways correlate with a decrease in neuronal injury in the two regions of brain most sensitive to ischemia, the striatum and hippocampus.

Materials and Methods

Animal Model

Newborn piglets, 3-4 days old, were obtained from Meck Swine, LLC (Refton, PA) and used within 4 days of receipt. The animals compared in our studies were from multiple litters. The piglets were anesthetized with 4% isoflurane, intubated and mechanically ventilated with air/30% oxygen mixture with a goal of maintaining normocapnia. Anesthesia was maintained with 1.5% isoflurane supplemented with fentanyl (30µg/kg bolus) and pancuronium (0.1mg/kg bolus). A femoral arterial catheter was placed to monitor mean arterial blood pressure (MAP), blood gases (PaCO₂, PaO₂), pH, hemoglobin, electrolytes and glucose concentrations using i-STAT blood gas machine (Abbot Point of Care Inc., Princeton, NJ). Electrocardiogram, nasopharyngeal and rectal temperature were measured throughout the study. At the end of each experiment, the piglets were perfused with heparinized saline and the brain was fixed in situ by perfusion with 10% buffered formalin phosphate and then harvested to quantify the extent of injury.

All animal procedures were carried out in strict accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Experimental Groups

Male newborn piglets were randomly assigned to one of three groups: 1) CPB with circulatory arrest (n=5, DHCA group), 2) DHCA with G-CSF at a concentration of 34 μ g/kg (n=5, G-CSF group) and 3) sham-operated (n=4). G-CSF was given by intravenous injection two hours prior the beginning of the CPB procedure. Treatment two hours before beginning the bypass procedure was considered appropriate for allowing the G-CSF to distribute to all tissues, including the brain. This timing is based on reports in the literature that a significant fraction of G-CSF crosses the blood brain barrier within an hour and the concentration in the extravascular space continues to increase for several hours [7, 17].

The sham-operated (control) animals did not undergo CBP and cooling but were anesthetized, intubated and mechanically ventilated in the same manner as the animals in the other groups

CPB Technique

The CPB circuit consisted of a Cobe Roller Pump (Cobe, Lakewood, CO), a membrane oxygenator (Lilliput 1, Dideco, Mirandola, Italy), arterial filter (Terumo Cardiovascular System Corp., Ann Arbor, MI) and Sarns Heater-Cooler System (Terumo Cardiovascular System Corp., Ann Arbor, MI). The circuit was primed with Plasmalyte-A (Baxter Healthcare Corp., Deerfield, Ill.) and 25% albumin. Donor whole blood was added to maintain a CPB hematocrit value of approximately 30%. Heparin (2000 units), fentanyl (50 μ g), pancuronium bromide (1 mg), calcium chloride (500 mg), dexamethasone (30 mg), cefazolin (500 mg), furosemide (3 mg), and sodium bicarbonate (25 mEq) were then added to the pump prime.

For the CPB procedure, median sternotomy was performed, 500U heparin was administered IV, and the ascending aorta and the right atrial appendage were cannulated. CPB flow rate was set at approx. 150 ml/kg/min. The piglets were then cooled with pH-stat blood gas management to a nasopharyngeal/brain temperature of 18°C over a 20-30 min period. The temperature-corrected arterial blood pH was maintained at 7.4 by addition of CO₂ (typically by adding 50 ml/min of CO₂ to the fresh gas flow of 1.5 l/min through the blood oxygenator). During cooling, the temperature was adjusted to keep the oxygenator/body temperature gradient no greater than 10°C. After the cooling period, the piglets were subjected to 60 min of DHCA. CPB was then resumed at 150 ml/kg/min and the piglets were rewarmed to 37 $\pm$ 1°C over 30 min. For all groups, ventilation was reinitiated 5 min before weaning from CPB. CPB was discontinued when body temperature reached 37°C. All animals received analgesia, paralysis, mechanical ventilation, and were continuously monitored throughout the recovery period of 8-9 hrs after weaning from CPB. No inotropes were used and the post-CPB arterial blood pressure was maintained with an infusion of saline/packed red blood cells to keep MAP above 50 mm Hg. At the end of each experiment, piglets were first perfused with heparinized saline and then with 10% phosphate-buffered

paraformaldehyde. The forebrain was extracted, post-fixed, cryoprotected in 30% sucrose and then cut into 10 μ m transverse sections. Every fifth section through the striatum and hippocampus was mounted.

TUNEL Assay

Apoptotic cells were visualized by *in situ* detection of DNA fragmentation (TUNEL) using NeuroTACS™ II *in situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD). Sections were counter-stained with Cresyl violet, cover slipped and analyzed by light microscopy. TUNEL-positive nuclei were counted within as many 1×1 mm counting boxes as could fit within an outline of the striatum and hippocampus of each section by a person not informed of the treatment. For each section, the density of TUNEL-positive cells was expressed as the number of apoptotic cells per mm². For each animal, the mean density of apoptotic cells was calculated in each region of the brain based on up to 10 sections.

Statistical Analysis

The numbers of apoptotic cells per mm² were determined for each brain region of interest and averaged within each animal. These measurements were then initially subjected to normality and equal variance tests. Following the determination that the data set met the criteria for normality and equal variance, the measurements were subjected to two-way ANOVA with the experimental group (treatment) and brain region considered as significant factors (SigmaPlot v. 12.3, San Jose, CA). Subsequently, data for each region were subjected to oneway ANOVA with the treatment considered as a significant factor. One-way ANOVA was then followed by individual *post hoc* comparisons between groups with Holm-Sidak correction for multiple comparisons. The results are presented as means $\pm$ standard deviation (SD), with differences between groups considered significant when the corrected for multiple comparisons p-values were lower than 0.05.

Results

Apoptotic cells were counted in TUNEL-stained sections, as shown in Figs. 1-3. The figures show representative images obtained from the striatum (Fig. 1) and the CA1 (Fig. 2) and CA3 (Fig. 3) regions of the hippocampus. For each region, the images of sections were prepared from a control (A), DHCA (B), and DHCA plus G-CSF treated (C) animal, and TUNEL-positive cells are marked with arrows. There were typically no or just 1 TUNEL-positive cell present in the control animals but in the DHCA group they were always present, with usually several TUNEL-positive cells per image.

To quantify the results, TUNEL-positive cells were counted as described in Materials and Methods and the results are presented in Figs. 4 A-C. Across all three treatments and regions, there was a highly significant effect of the treatment ($F_{2,2,39}=30.6$, $p<0.001$; two-way ANOVA with interactions), whereas neither the effect of the region nor the interaction between the treatment and region were significant ($p=0.071$ and $p=0.28$, respectively). Subsequent analysis within each region using one-way ANOVA confirmed strong effects of the treatment within each region. Specifically, individual inter-group comparisons with Holm-Sidak correction revealed that the numbers of injured cells in each of the three

analyzed regions were significantly higher in the DHCA than in the control group. In the striatum, the number of injured cells increased from 0.46 ± 0.37 (n=4) to 3.67 ± 1.57 (n=5) ($p=0.002$). In the CA1 region of the hippocampus, the number of injured cells increased from 0.11 ± 0.19 (n=4) to 5.16 ± 1.57 (n=5, $p=0.001$), and in the CA3 region, from 0.28 ± 0.25 (n=4) to 2.98 ± 1.82 (n=5, $p=0.040$).

Injection of G-CSF prior to the CPB procedure significantly reduced the density of injured cells in the striatum and CA1 region of the hippocampus when compared to the DHCA treatment alone. In the striatum, injection of G-CSF decreased the number of injured cells from 3.67 ± 1.57 to 1.88 ± 0.63 (n=5) ($p=0.039$), and in the CA1 region, the density of injured cells decreased from 5.16 ± 1.57 to 3.24 ± 0.88 (n=5) ($p=0.024$). The latter measurement was still significantly higher than in the control group ($p=0.007$; Fig. 4.B). Thus, pretreatment with G-CSF reduced the density of apoptotic cells by 51% in the striatum and by 37% in the CA1 region when compared to the DHCA treatment only. The difference between the DHCA only and DHCA with G-CSF pretreatment groups was not significant in the CA3 region (2.98 ± 1.82 vs. 2.15 ± 0.69 (n=5), $p=0.31$), but it is of note that apoptotic cell density in this region did not differ significantly between the DHCA with G-CSF and control groups. This was in contrast to the significant difference between the DHCA only and control groups (above and Fig. 4.C), consistent with a moderate ameliorating effect of G-CSF on CPB-elicited apoptotic cell injury in the CA3 region.

The number of injured cells in each field was low because the measurements were made after a relatively short time of recovery (8-9hrs).

Discussion

The goal of this study was to determine whether treatment with G-CSF prior to cardiopulmonary bypass-deep hypothermic circulatory arrest (CPB-DHCA)-mediated ischemic insult would decrease neuronal injury in the brain. Our injury model is significantly different from most models of hypoxic/ischemic injury presented in literature. Although the brain is hypoxic for a substantial period of time, the temperature during that period is far below normal. This makes it problematic to extrapolate to normothermic models because low temperature plays a significant role in protecting the brain from hypoxic-ischemic injury [18, 19].

On the other hand, this model also is unique in the fact that the onset of hypoxia can be predicted. This allows for the G-CFS to be injected at a specific time, in this case prior to beginning of the insult, rather than post insult.

DHCA, with variable exposure times, is used during surgical intervention of neonates with congenital heart disease (CHD). CHD is a common birth defect, affecting 8 per 1000 live births. Today, thanks to improved perfusion techniques, better pharmacology and postoperative care, even the most complex surgical repairs are performed with low operative mortality. Therefore, the focus of physicians and researchers has shifted towards the long-term outcomes in these patients, particularly neurodevelopmental progress and quality of

life. Multiple studies have demonstrated neurodevelopmental dysfunction (NDD) in patients with complex CHD.

The prevalence of impaired neurologic function appears to vary, depending on the specific cardiac diagnosis [20,21]. Long-term follow up studies in these children have revealed distinctive patterns of NDD characterized by cognitive impairment, impaired executive function, expressive speech and language abnormalities, impaired visual-spatial and visual-motor skills, attention deficit/hyperactivity disorder, motor delays, and other learning disabilities [22-34].

Depending on the circumstance and the degree of injury, brain ischemia may be a contributing factor to this impairment in neurologic function.

Advances in the treatment of CPB-DHCA hypoxic/ischemic brain injury and finding possible protective compounds will only evolve from a thorough understanding of the neuropathological processes involved in neuronal survival and death. Based on our previous studies and data of other investigators, following DHCA, the primary mechanisms for cell death in the newborn and mature brain involve apoptotic and inflammatory signaling pathways [19, 35-39].

Striatum and hippocampus are regions of the brain very vulnerable to ischemic injury [40-43]. Therefore, our studies have focused primarily on those areas. The results show that prolonged DHCA causes significant injury in the striatum and CA1 region of the hippocampus. Considerably fewer injured cells were observed in the CA3 region of the hippocampus. These results are in agreement with findings of other investigators that the CA3 region of the hippocampus is much less sensitive to hypoxia than CA1 [44-47].

The observed numbers of injured cells in the striatum and CA1 region of the hippocampus are significantly diminished by single injection of G-CSF prior to bypass. This finding is important and clinically relevant. DHCA-mediated injury, as described above, can cause major impairment of neurologic function, including motor function and memory. Brain regions predominantly responsible for these functions are the striatum and hippocampus [48, 49].

What are possible some of the potential mechanisms of G-CSF neuroprotection in the striatum and hippocampus? As was stated in the introduction, the neuroprotective actions of G-CSF in different models of hypoxia and ischemia have been attributed to its anti-inflammatory and anti-apoptotic properties [7, 12-14]. Our earlier study showed that pretreatment with G-CSF prior to bypass diminished pro-apoptotic and increased anti-apoptotic signaling (increase in pAkt and Bcl-2) in the striatum and hippocampus [15]. This strongly suggests that anti-apoptotic properties of G-CSF could be at least partly responsible for the protective effect of G-CSF observed in the present study.

The exact mechanisms of G-CSF neuroprotection require future investigation but it is clear that several relevant signaling pathways are affected by G-CSF. The anti-apoptotic activity of G-CSF on neurons may be mediated at least partially by the PI3K/Akt pathway [50-52]. Protein kinase B (Akt), a downstream target of PI3K, is a critical anti-apoptotic factor in

controlling the balance between survival and apoptosis in multiple cell systems, including neurons. This involves several mechanisms, including phosphorylation of the Bcl-2-associated death protein and caspase-9 and induction of Bcl-2 expression [51, 53-57]. In our earlier study, we showed that injection of G-CSF, prior to DHCA, led to the increased Bcl-2, Bcl-2/Bax ratio and expression of pAkt in the striatum and entire hippocampus [15].

Akt activity may be also responsible for the different vulnerability to ischemia-hypoxia of CA1 and CA3 regions of the hippocampus. Jackson et al. [44] suggested that regional hippocampal differences in the Akt pathway may contribute to regional differences in neuronal vulnerability and help protect the CA3 region. They reported that cell survival signaling, as measured by activated Akt, was significantly lower in the CA1 region when compared with the CA3. Therefore, it is reasonable to propose that increase in Akt activity in the striatum and CA1 region of the hippocampus plays a major role in G-CSF-stimulated brain protection following DHCA surgery.

Another factor that may be contributing to the different sensitivity of CA1 and CA3 regions of the hippocampus to hypoxia/ischemia, and may be affected by G-CSF, is a difference in the concentration of excitatory amino acid receptors. When compared to the CA3 region, the CA1 region has a higher concentration of glutamate, as well as NMDA receptors, that play an important role in neuronal injury [58-60].

Schäbitz et al. [51] reported that G-CSF may have excitoprotective properties that are involved in neuroprotection and, therefore, the effect of G-CSF will occur predominantly in the CA1 region of the hippocampus.

Similar mechanisms of neuroprotection to the ones proposed for the CA1 region of the hippocampus, such as increased Akt expression and decreased excitotoxicity of glutamate may also be proposed for the striatum. However, there are probably also some differences in the mechanisms of protection by G-CSF between the hippocampus and striatum. Striatum is a dopaminergic region and the levels of dopamine are much higher there than in other brain regions, including the hippocampus. Evidence for a role of dopamine in mediating ischemic cell death is well established [61]. Globus et al. [62] and Filloux and Wamsley [63] reported that a lesion at the substantia nigra has a neuro-protective effect on the striatum, and that this is related to the inhibition of dopamine release. Dopamine may have a direct neurotoxic effect on neuronal cells, may potentiate neuronal damage by its effects on the glutaminergic system, and may increase production of free radicals. [64]

Hoyt et al. [65], Porat and Simantov [66] and Noh et al. [67] have reported that high levels of dopamine can cause apoptosis.

We previously reported [68] that the levels of extracellular dopamine do not change during the first 30 min of DHCA but following this time period those levels increase rapidly. The time of onset of the excessive release of dopamine corresponds with end of the generally accepted “safe” period of DHCA. The release of dopamine is likely to be an important factor in high sensitivity of the striatum to ischemic/hypoxic injury. A significant role of dopamine in the striatal, but not hippocampal, injury has been suggested by Marie et al. [69]. The authors evaluated rat brain 72 hrs after ischemia (four vessel occlusion) and reported that α -

methyl-*p*-tyrosine (AMT) treatment significantly decreased neuronal necrosis in the striatum but had no cytoprotective effect in the CA1 region of the hippocampus. AMT is an inhibitor of dopamine synthesis and, in our earlier studies, we have shown that pretreatment of piglets with this compound decreased dopamine level in the striatum by about 80% compared to controls [70-72]. Liew et al. [73] reported that, in rats, G-CSF treatment decreased release of dopamine in the ischemic striatum. Therefore, we may deduce that an important mechanism for G-CSF protection from DHCA-mediated ischemic injury in the striatum is also through the suppression of dopamine release.

As stated above, the exact mechanisms of G-CSF neuroprotection requires further investigation, but it is clear that this compound has a substantial protective effect and that the mode of this protection involves Akt and neurotransmitters such as dopamine and glutamate. There are no reported negative effects of G-CSF treatment, certainly at the dose used in our studies. This makes pre-treatment with G-CSF a very promising approach for obtaining better neurologic outcomes following CPB procedures.

ACKNOWLEDGEMENTS

The research was supported by a grant HL-58669 from the National Institutes of Health, Bethesda, MD, USA.

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Figure 1.

Effect of DHCA and G-CSF on number of TUNEL positive cells in striatum (Fig. 1A-C), the CA1 region of hippocampus (Fig. 2A-C) and CA3 region of hippocampus (Fig. 3A-C). Each figure shows representative images of TUNEL stained sections of tissue from Control (A), DHCA treated (B), and DHCA + G-CSF (C) animals. The TUNEL positive cells are marked with arrows and the inset is a 4x increased magnification of the indicate region of the image. The magnification is indicted by a 200 µm-long bars.

Figure 2.

Effect of DHCA and G-CSF on number of TUNEL positive cells in striatum (Fig. 1A-C), the CA1 region of hippocampus (Fig. 2A-C) and CA3 region of hippocampus (Fig. 3A-C). Each figure shows representative images of TUNEL stained sections of tissue from Control (A), DHCA treated (B), and DHCA + G-CSF (C) animals. The TUNEL positive cells are marked with arrows and the inset is a 4x increased magnification of the indicate region of the image. The magnification is indicted by a 200 µm-long bars.

Figure 3.

Effect of DHCA and G-CSF on number of TUNEL positive cells in striatum (Fig. 1A-C), the CA1 region of hippocampus (Fig. 2A-C) and CA3 region of hippocampus (Fig. 3A-C). Each figure shows representative images of TUNEL stained sections of tissue from Control (A), DHCA treated (B), and DHCA + G-CSF (C) animals. The TUNEL positive cells are marked with arrows and the inset is a 4x increased magnification of the indicate region of the image. The magnification is indicted by a 200 µm-long bars.

Figure 4.

Quantification of the effects of DHCA only and DHCA with G-CSF pretreatment on the density of TUNEL-positive (apoptotic) cells in the striatum (A), CA1 region of hippocampus (B) and CA3 region of hippocampus (C). The bars show the mean density of TUNEL-positive nuclei found within each region of interest per mm² of tissue. For each animal, the mean density of apoptotic cells was calculated for each region based on cell counts obtained from up to 10 different tissue sections. The bars show the mean density of TUNEL-positive nuclei found within each region of interest calculated per mm² of tissue for 4 control animals, 5 animals in the DHCA group, and 5 animals in the DHCA + G-CSF group. In each region, DHCA group had significantly elevated density of apoptotic cells when compared to the control group, and pretreatment with G-CSF significantly reduced the apoptotic effect of DHCA procedure in the striatum and the CA1 region. Significance levels for all comparisons were determined using one-way ANOVA with Holm-Sidak correction for multiple comparisons.