Heme oxygenase-1 induction contributes to renoprotection by G-CSF during rhabdomyolysis-associated acute kidney injury

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Rhabdomyolysis is a clinical condition initiated by acute disruption of skeletal muscle due to physical or chemical damage from crush injury, exhausting exercise, surgery, or toxins (10). A notable complication associated with rhabdomyolysis is acute kidney injury (AKI), resulting in rapid deterioration of renal function or acute renal failure (35, 41). Rhabdomyolysis-associated AKI involves the nephrotoxicity of heme, which is released from myoglobin in a pathogenic quantity. Experimentally, rhabdomyolysis-associated AKI is modeled in vivo in animals by glycerol disruption of skeletal muscle and in vitro by hemin/heme treatment of renal tubular cells (7, 19, 20). These studies have not only characterized rhabdomyolysis-associated tissue damage and renal cell injury but have also led to the discovery of heme oxygenase-1 as a critical renoprotective molecule.

Heme oxygenases are the rate-limiting enzymes that catalyze the degradation of heme, releasing biliverdin, iron, and carbon monoxide (4, 13, 14, 18, 29). Among the three isoforms, heme oxygenase-1 (HO-1) is inducible in response to cellular stress. Importantly, upon induction, HO-1 protects against tissue and cell injury. In kidneys, the protective effects of HO-1 have been demonstrated in a variety of renal injury models including ischemia-reperfusion, glycerol-induced rhabdomyolysis, cisplatin nephrotoxicity, and endotoxemia (19, 20, 26–28, 36).

Granulocyte colony-stimulating factor (G-CSF) is a pleiotropic cytokine which is well known for its ability to stimulate the mobilization, proliferation, and probably differentiation of bone marrow cells. In kidneys, ischemia-reperfusion induces G-CSF in serum and renal tissues (42). Interestingly, depending on the dosage and time of application, G-CSF has been shown to protect against AKI in experimental models of ischemia-reperfusion and nephrotoxins (5, 12, 16, 21, 31, 33). Despite these observations, the mechanism(s) underlying the renoprotective effects of G-CSF is largely unclear (22). The beneficial effects of G-CSF may be related to bone marrow cell mobilization into renal tissues. However, during AKI the mobilized bone marrow cells do not mainly function to differentiate into renal tubules (3, 11, 17); instead, they may produce trophic factors, contributing to renal tissue repair or regeneration (1, 32, 34). In addition, G-CSF may prevent tissue damage by directly acting on parenchymal cells. It has been reported recently that G-CSF can protect cardiomyocytes from oxidative stress injury and neurons against glutamate toxicity injury (8, 23). Whether G-CSF has directly cytoprotective effects in renal cells or tissues is unclear (16, 30).

In the current study, we have demonstrated evidence for the renoprotective effects of G-CSF in experimental models of rhabdomyolysis-associated AKI. G-CSF mobilizes and recruits bone marrow cells into renal tissues, but the cells do not seem to differentiate into renal tubules. Notably, G-CSF induces HO-1, a well-recognized renoprotective protein. Inhibition of HO-1 attenuates the renal-protective effects of G-CSF, suggesting an important role for HO-1 in tissue protection by G-CSF.

MATERIALS AND METHODS

Mouse model of rhabdomyolysis-associated AKI. C57BL/6 mice (male, 8–10 wk) were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility of the Charlie Norwood Veterans Affairs (VA) Medical Center (Augusta, GA). The animals were housed with a 12:12-h light-dark cycle and food and water available ad libitum. To induce rhabdomyolysis, the animals were injected with 7.5 ml/kg 50% glycerol intramuscularly to the two hindlegs or injected with saline as a control (20). To study the effects of G-CSF, 200 mg/kg−1·day−1 recombinant human G-CSF (Amgen, Thousand Oaks, CA) or the same volume of saline was administrated intraperitoneally to the animals for 5 consecutive days before a glycerol injection. To inhibit HO-1, 20 mg/kg protoporphyrin IX
Zn(II) (ZnPP) was administered intraperitoneally to the mice 2 h before the glycerol injection, while saline was given to the no-ZnPP animals. All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committees of the Charlie Norwood VA Medical Center.

Analysis of renal function and histology. Renal function and histology were examined as previously (2, 38–40). Blood urea nitrogen (BUN) was monitored to indicate renal function. Briefly, blood samples were collected to obtain serum following clotting and centrifugation. BUN was measured with a kit from Biotron Diagnostics (Hemet, CA). To analyze renal histology, kidneys were harvested from the animals, fixed with 4% paraformaldehyde, and paraffin-embedded. Tissue sections of 4 μm were subjected to hematoxylin-eosin (H&E) staining and examined by microscopy. Tubular damage was indicated by necrotic lysis, tubular dilation, cast formation, and sloughing of cellular debris into the tubular lumen. Tubular injury scores were determined by the percentage of renal tubule injury: 0, no damage; 1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75%. Images of representative fields were also recorded.

Terminal transferase-dUTP nick-end labeling assay. Terminal transferase-dUTP nick-end labeling (TUNEL) assay was performed to detect apoptosis using the in situ Cell Death Detection kit from Roche Applied Sciences (Indianapolis, IN) as described previously (38, 39). Briefly, cells and kidney tissues were fixed in 4% paraformaldehyde and permeabilized in sodium citrate, pH 6.0. The samples were then incubated with TM red-labeled dUTP and TUNEL-positive nuclei were identified by fluorescence microscopy.

Immunohistochemical staining of HO-1 in renal tissues. Renal tissues were fixed with 4% paraformaldehyde, paraffin-embedded, and cut into 4-μm sections. The tissue sections were then deparaffinized and permeabilized by 2 h of incubation at 65°C in 0.1 M sodium citrate, pH 6.0. The tissues were subsequently incubated for 1 h with 0.1% phenylhydradine and 0.03% H2O2 at 37°C to block the endogenous peroxidase activity. After that, the tissues were incubated sequentially with a blocking buffer (2% BSA, 0.2% nonfat dry milk, 0.8% Triton X-100, and 2% normal donkey serum) and the anti-HO-1 antibody (1:200 dilution, Stressgen, Victoria, BC) in the blocking buffer. After incubation with biotin-labeled secondary antibody, the color was developed with an ABC kit from Vector Laboratories (Burlingame, CA). The staining was evaluated in a blinded manner.

Caspase activity assay. The activity of caspases was measured as described previously (2, 38, 39). Briefly, cells were treated with 1% Triton X-100 and added to (25 μg of protein/sample) an enzymatic reaction containing 50 μM carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD.AFC), a fluorogenic substrate for caspase-3, -6, and -7. Fluorescence at 360-nm excitation/530-nm emission was measured. For each measurement, a standard curve was constructed using free AFC. Based on the standard curve, the fluorescence reading from each enzymatic reaction was translated into nanomolar free AFC μg protein−1 h−1 to indicate caspase activity.

Immunoblot analysis. Whole cell lysate and tissue lysate were collected in a buffer containing 2% SDS. Reducing gel electrophoresis and protein transferring/blotting were conducted by standard procedures. The blots were incubated with 5% fat-free milk for blocking and then exposed to specific primary antibodies overnight at 4°C. Finally, the blots were exposed to the horseradish peroxidase-conjugated secondary antibody, and antigens on the blots were revealed using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL). Polyclonal anti-HO-1 from Stressgen, anti-G-CSF receptor from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal anti-β-actin (Sigma) antibodies were used at 1:2,000, 1:200, and 1:5,000 dilutions, respectively, for immunoblotting in this study.

RT-PCR analysis of mRNA expression. Total RNA were extracted with TRI reagent (Sigma-Aldrich). RT-PCR was conducted by standard methods using the Superscript First-strand synthesis system (Invitrogen, Carlsbad, CA). The primers for amplification of HO-1 were 5′-CAGTCTATGCCGGCCTCTAC-3′ (forward) and 5′-AC-CAGCAGCTCAGGATGT-3′ (reverse). The primers for β-actin gene were 5′-GTGGGCGGCCCAAGGCACCA-3′ (forward) and 5′-CTCCCTTAAATGCCAGCATTTTC-3′ (reverse).

RESULTS

G-CSF pretreatment ameliorates rhabdomyolysis-associated AKI. To examine the effects of G-CSF on rhabdomyolysis-associated AKI, male C57BL/6 mice were pretreated for 5 consecutive days with G-CSF or saline as a control. The animals were then injected with 7.5 mg/kg 50% glycerol in the hindleg muscle. Serum samples were collected from the animals to measure BUN to indicate renal function. As shown in Fig. 1A, without a glycerol injection both saline- and G-CSF-treated animals showed low baseline levels of BUN on day 0. One day after the glycerol injection, BUN was increased from 34.9 ± 6.3 to 187.3 ± 27.2 mg/dl (n = 10) in the saline-pretreated group and further elevated to 198.9 ± 30.3 mg/dl (n = 6) at day 2. The glycerol-induced BUN elevation was partially but significantly ameliorated in the animals pretreated with G-CSF. As a result, the BUN values in these animals were 145.8 ± 23.9 and 135.6 ± 57.0 mg/dl (n = 7) at days 1 and 2 of glycerol treatment, respectively (Fig. 1A).

The renoprotective effect of G-CSF following the glycerol injection was also confirmed by the examination of renal histology. As shown in Fig. 1B, in the absence of a glycerol injection, renal tissues showed a normal healthy histology regardless of G-CSF pretreatment. However, 2 days after the glycerol injection, widespread tissue damage was noticed in the kidneys. Tissue damage was shown in both the cortex and renal medulla. The injured tubules showed the loss of microvilli, necrotic lysis, tubular dilation, cast formation, or sloughing of cellular debris into the tubular lumen. However, it is noteworthy that severe damage including necrotic lysis was
mainly in the outer stripe of outer medulla and cortex, whereas the injurious changes in the inner medulla were mainly cast formation and tubular dilation. In mice pretreated with G-CSF, glycerol-induced renal tissue damage was partially ameliorated (Fig. 1B). We further graded the renal tissue damage in three separate experiments. A summary of the tubular damage scores is shown in Fig. 1C (n = 6). Clearly, G-CSF could partially but significantly attenuate renal tissue damage during glycerol-induced rhabdomyolysis. Of note, the protective effect of G-CSF in the outer medulla was not as evident as that in the cortex and inner medulla. One explanation is that the S3 proximal tubules in the outer stripe of the outer medulla were severely injured and harder to protect. We also examined apoptosis in the tissues by TUNEL assay. Glycerol induced 7.1 ± 3.6 apoptotic cells/mm² tissue, which was reduced to 4.5 ± 3.2 cells/mm² by G-CSF pretreatment (n = 5).

Beneficial effects of G-CSF on animal survival following glycerol-induced rhabdomyolysis. We further monitored animal death/survival following the glycerol injection. As shown in Fig. 2, animal death started in the saline-pretreatment group after 2 days of glycerol treatment and, by the end of 10 days, only ~30% animals were alive (n = 14). In contrast, G-CSF-pretreated animals (n = 7) did not die until day 6 after the glycerol injection. This group of animals had a >70% survival rate during the whole observation period. The difference between the survival rates of these two groups is significant by Kaplan-Meier analysis (P < 0.05). Thus G-CSF not only postponed animal death but also improved long-term survival following glycerol-induced rhabdomyolysis.

G-CSF pretreatment inhibits hemin-induced apoptosis in cultured tubular cells. A major mediator or inducer of renal injury during rhabdomyolysis is the free heme that is released from myoglobin from disrupted muscles. Thus hemin treatment of cultured tubular cells has been established to mimic the in vivo situation of rhabdomyolysis-associated renal injury (7). Using a similar model, we tested whether G-CSF could directly protect against hemin-induced apoptosis in cultured RPTC. As shown in Fig. 3A, typical apoptosis was induced during hemin treatment, showing cellular shrinkage, nuclear condensation, and fragmentation. These cells were also positive for TUNEL staining (Fig. 3A, bottom). By cell counting, 50 μM hemin induced ~30% apoptosis at 6 h, which increased to ~50% at 10 h. Notably, the apoptosis was reduced by 500 ng/ml G-CSF pretreatment to 25 and 30% at 6 and 10 h, respectively (Fig. 3B). As a biochemical marker of apoptosis, we measured caspase activity. Hemin induced an over fivefold increase in caspase activity within 6 h, which was partially but significantly suppressed by G-CSF (Fig. 3C). The effect of G-CSF on...
As shown in Fig. 5A, G-CSF treatment (200 mg·kg\(^{-1}\)·day\(^{-1}\)) of mice for 5 days led to a marked increase in HO-1 in the renal cortical tissue lysate. By immunohistochemical staining, we showed that HO-1 was mainly induced in cortical tubules and not in glomeruli (Fig. 5B).

Inhibition of HO-1 by ZnPP attenuates the protective effects of G-CSF. To determine whether HO-1 induction contributed to the observed renoprotective effects of G-CSF, we examined the effects of ZnPP, a pharmacological inhibitor of HO-1 (15, 25). RPTC cells were cultured for 3 days in the absence or presence of 500 ng/ml G-CSF for pretreatment. One group of dishes was then incubated with 2 \(\mu\)M ZnPP for 2 h to inhibit HO-1 (Fig. 6A), while the other group received the vehicle solution. Both groups were finally treated with 50 \(\mu\)M hemin for 8 h to induce apoptosis. As shown in Fig. 6A, hemin treatment induced \(\approx\)50% apoptosis in RPTC cells without G-CSF pretreatment, which was decreased to 30% by G-CSF pretreatment. ZnPP alone did not induce much apoptosis; however, it significantly enhanced apoptosis during hemin treatment. Notably, ZnPP attenuated the protective effects of G-CSF and, as a result, the cells with or without G-CSF pretreatment showed similarly high levels of apoptosis, \(>70\%\), suggesting that HO-1 induction was critical to the renoprotective effects of G-CSF in vitro.

We further determined the effects of ZnPP in vivo in C57BL/6 mice. To this end, the animals were divided into three groups. Group 1 received saline, while groups 2 and 3 were pretreated with G-CSF for 5 days. Two hours before the glycerol injection, group 3 was administrated with 20 mg/kg ZnPP, whereas groups 1 and 2 were given the vehicle solution. BUN was measured at days 0, 1, and 2 after the glycerol injection to indicate renal injury. As shown in Fig. 6B, following the glycerol injection the mice in group 1 (No G-CSF) showed BUN increases at both days 1 and 2, to \(\approx\)110 mg/dl. The BUN increases were significantly suppressed by G-CSF pretreatment in group 2 (G-CSF), to 65 mg/dl. However, in group 3 the renoprotective effects of G-CSF were diminished by ZnPP (Fig. 6B).

**DISCUSSION**

The effects of G-CSF have been studied in experimental models of AKI induced by ischemia-reperfusion, folic acid, and cisplatin toxicity. In the majority of the studies, G-CSF was shown to mobilize bone marrow cells into renal tissues and ameliorate renal injury (5, 12, 21, 31). However, bone marrow cell mobilization-associated granulocytosis by G-CSF may also worsen renal injury as shown in a mouse model of renal ischemia-reperfusion injury (33). In the current study, we have determined the effects of G-CSF on rhabdomyolysis-associated AKI. Our results show that G-CSF protects against glycerol-induced AKI in vivo and hemin-induced apoptosis in vitro. Mechanistically, we have demonstrated the first evidence for HO-1 induction by G-CSF. HO-1, after being induced, contributes to the renoprotective effects of G-CSF. Thus the results have revealed a novel mechanism of tissue protection by G-CSF.

As a pleiotropic cytokine, G-CSF is well known for its ability to stimulate the mobilization and proliferation of bone marrow cells. During AKI induced by renal ischemia and cisplatin nephrotoxicity (12, 30, 33), G-CSF mobilizes bone marrow cells into injured tissues. Consistently, using a chimerical mouse model with transplanted GFP-positive bone marrow, we showed that G-CSF increased the number of bone...
marrow cells in the renal cortex during glycerol-induced AKI (Supplemental Fig. S3). Despite these findings, it remains unclear whether and how the bone marrow cells contribute to renal injury and repair or regeneration. Although bone marrow stem cells may differentiate into renal tubular cells, recent studies indicate that bone marrow cell differentiation into or fusion with tubular cells is not frequent but a rare event (3, 11, 17, 30). In our study, GFP-labeled bone marrow was reconstituted into bone marrow-ablated recipient mice. Although GFP-labeled cells were detected in the renal tissues of the recipient mice, these cells were shown in the interstitium and not incorporated into renal tubules. G-CSF pretreatment increased the recruitment of bone marrow cells into renal tissues but did not induce the differentiation or

Fig. 3. Effects of G-CSF on hemin-induced apoptosis in rat proximal tubule cells (RPTC) cells. RPTC cells were preincubated for 3 days with or without 500 ng/ml G-CSF. The cells were then treated with 50 μM hemin for 6 or 10 h, or left untreated as control. A: representative cell morphology after 6 h of treatment. Cells were subjected to Hoechst 33342 or TUNEL staining and then examined by phase-contrast and fluorescence microscopy. B: % apoptosis. The cells with typical apoptotic morphology (cellular shrinkage, formation of apoptotic bodies, and nuclear fragmentation) were counted to determine the percentage of apoptosis. C: caspase activity. Cell lysate was collected to determine caspase activity by an enzymatic assay as described in MATERIALS AND METHODS. Values are means ± SD (n = ≥3). The results demonstrate cytoprotective effects of G-CSF on hemin-induced tubular cell apoptosis. *Significantly different from the control groups without hemin treatment (P < 0.05). #Significantly different from the hemin-treated groups without G-CSF pretreatment (P < 0.05).
incorporation of these cells into renal tubules for tissue repair (Supplemental Fig. S3).

On the other hand, bone marrow cells recruited into renal tissues may provide a protective, reparative, or regenerative microenvironment. This has been demonstrated recently for mesenchymal/stromal stem cells, a specific stem cell population in bone marrow. In an experimental model of renal ischemia-reperfusion, Togel et al. (34) showed that infused mesenchymal/stromal stem cells can protect against kidney injury by their vasculotropic, paracrine actions. In a separate study, Bi et al. (1) showed that intravenous infusion or intraperitoneal injection of bone marrow-derived stromal cells can enhance tubular cell proliferation and decrease tubular cell apoptosis, leading to renoprotection during cisplatin-induced AKI. Importantly, similar protective effects were demonstrated for conditioned media from cultured stromal cells (1). Together, these studies suggest that mesenchymal/stromal stem cells recruited into injured renal tissues may protect the kidneys by secreting protective and/or regenerative factors.

In our study, G-CSF treatment led to significantly higher mobilization and recruitment of bone marrow cells into injured renal tissues, increasing a tropic cell population for renal protection and repair.

Interestingly, we have also suggested a direct cytoprotective action of G-CSF in renal tubular cells. In 2004, Gonzales-Michaca and colleagues (7) established an in vitro model to study rhabdomyolysis-associate AKI by hemin incubation of proximal tubular cells. In this model, hemin induced apoptosis as shown by cell morphology, TUNEL staining, and caspase activation (7). Using a similar model, we now demonstrate that G-CSF pretreatment can suppress hemin-induced apoptosis in RPTC cells (Fig. 3). While the mechanism underlying the direct cytoprotection remains unclear, hemin-induced caspase activation is suppressed, suggesting that the antiapoptotic effects of G-CSF result from the inhibition of caspase-activation pathways. In addition, our results show that RPTC cells express the G-CSF receptor (Fig. 4), although it remains to be determined whether the cytoprotective effects of G-CSF depend on G-CSF receptor-mediated signaling. A direct cytoprotective action of G-CSF has been reported in other cell types. For example, Harada et al. (8) showed that G-CSF can bind to its receptor on cardiomyocytes and induce phosphorylation and activation of the Jak 2/Stat 3 pathway, leading to expression of the antiapoptotic protein Bcl-2 and myocardial protection. More recently, Nishio et al. (23) reported that G-CSF can protect against glutamate-induced cell death in cultured neurons.

Regarding the cytoprotective mechanisms of G-CSF, we have identified an intriguing induction of HO-1. HO-1 is the inducible form of heme oxygenase, the rate-limiting enzyme in heme degradation. It has now been increasingly appreciated that HO-1 has a much broader role in tissue injury and protection, including antiapoptotic and anti-inflammatory actions (4, 14, 18, 29). In our study, HO-1 was induced by G-CSF in vitro in cultured tubular cells and also in vivo in...
mouse kidneys. Notably, pharmacological inhibition of HO-1 by ZnPP could attenuate the renoprotective effects of G-CSF (Fig. 6), suggesting that HO-1 induction is important to G-CSF-mediated protection. We showed that G-CSF induced both HO-1 mRNA and protein, indicating the involvement of transcriptional mechanisms; however, further studies need to gain more mechanistic insights about the regulation and identify the responsible transcription factors.
In conclusion, we have demonstrated the renoprotective effect of G-CSF in experimental models of rhabdomyolysis-associated AKI. The protection may not only be related to bone marrow cell mobilization but also to direct cytoprotective effects of G-CSF. A novel cytoprotective mechanism of G-CSF revealed by our current study is the induction of HO-1.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


