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Modulation of the HGF/c-Met axis impacts prolonged hematopoietic progenitor mobilization following trauma and chronic stress

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Abstract

Background: Trauma and hemorrhagic shock trigger mobilization of hematopoietic progenitor cells (HPC) from bone marrow to peripheral blood. Hepatocyte growth factor (HGF), tyrosine-protein kinase Met (c-Met), matrix metalloproteinase 9 (MMP-9), and corticosterone regulate this mobilization process. We hypothesized that beta-blockade with propranolol and sympathetic outflow inhibition with clonidine following trauma and chronic stress would decrease hematopoietic progenitor cell mobilization.

Methods: Sprague-Dawley rats were randomized to undergo three models of injury and stress: lung contusion (LC), LC plus hemorrhagic shock (LCHS), or LCHS plus chronic restraint stress for 2h daily (LCHS/CS). Propranolol and clonidine were administered by daily intraperitoneal injection until sacrifice on day seven. Bone marrow HGF, c-Met, and MMP-9 were measured by real-time PCR. Plasma corticosterone was measured by ELISA. %HPC in peripheral blood was measured by flow cytometry.

Results: Propranolol and clonidine significantly decreased bone marrow MMP-9 expression, plasma corticosterone levels, and HPC mobilization, and significantly increased hemoglobin levels. HPC mobilization was greatest following LCHS/CS (5.4 ± 1.8) and was significantly decreased by propranolol (2.2 ± 0.9 , $p < 0.001$) and clonidine (1.7 ± 0.5 , $p < 0.001$). Hemoglobin (g/dL) was lowest following LCHS/CS (12.3 ± 1.2) and was significantly increased by propranolol (13.7 ± 0.4 , $p = 0.022$) and clonidine (14.1 ± 1.1 , $p < 0.001$).

Conclusions: Severe injury was associated with increased bone marrow HGF, c-Met, and MMP-9, circulating corticosterone, HPC mobilization, and persistent anemia. Attenuating the neuroendocrine response to injury and stress with propranolol and clonidine reduced MMP-9 expression, corticosterone levels, HPC mobilization, and the degree of anemia.

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Keywords

Trauma; stress; hematopoietic progenitor mobilization; hepatocyte growth factor; tyrosine-protein kinase Met

Introduction

Following traumatic injury and hemorrhagic shock, there is increased mobilization of hematopoietic progenitor cells (HPC) from the bone marrow to peripheral blood and sites of injury (1, 2). This process appears to be necessary for wound healing and tissue repair, but prolonged HPC mobilization and loss of these progenitor cells from the bone marrow is also associated with bone marrow dysfunction and anemia (3, 4). Activation of the granulocyte colony-stimulating factor (G-CSF)/stromal cell derived factor-1 (SDF-1) axis hones HPCs to the site of injury (5, 6). Early mobilization of HPCs following traumatic injury and hemorrhagic shock has been associated with elevation of plasma G-CSF 24 hours after injury, and exogenous administration of G-CSF and SDF-1 to rats subjected to traumatic injury and hemorrhagic shock has been shown to increase HPC mobilization to injured tissue five days after injury (5, 6).

Our understanding of post-traumatic HPC mobilization remains incomplete. Downstream of the G-CSF/SDF-1 axis, hepatocyte growth factor (HGF), tyrosine-protein kinase Met (c-Met), matrix-metalloproteinase-9 (MMP-9) and circulating corticosterone may each play a role in this process. HGF is produced by bone marrow leukocytes in response to G-CSF/SDF-1 activation and interacts with c-Met, its receptor on HPCs. HGF/c-Met interaction is a process that may be best known for its role in propagating malignant potential and promoting stem and progenitor cell homing following bone marrow transplantation, but has also been implicated in stress-induced HPC mobilization (4, 7, 8). Tesio et al.(4) demonstrated that G-CSF induces c-Met expression and signaling leading to HPC mobilization, and that c-Met inhibition decreases HPC mobilization by interfering with chemotactic migration toward SDF-1 in a process mediated by reactive oxygen species signaling. MMP-9, produced by bone marrow leukocytes and HPCs in response to G-CSF activation and pro-inflammatory cytokines, also appears to be an important mediator of stress-induced HPC mobilization (9, 10). Kollet et al.(10) found that this process occurs in both murine and human HPC mobilization, demonstrating that neutralization of the SDF-1 receptor CXCR4 disrupted this process and that exogenous SDF-1 restored mobilization, suggesting causality. Circulating corticosterone, under the direct influence of the neuroendocrine stress response, has also been shown to promote hematopoietic stem cell mobilization (11). Pierce et al.(11) reported that corticosterone promotes hematopoietic stem cell mobilization by upregulating actin-organizing molecules and interacts with the Nr3c1 glucocorticoid receptor, which is nearly ubiquitous on human cells and influences several stress-response pathways.

The effects traumatic injury and chronic stress on HGF, c-Met, MMP-9, and corticosterone have not been reported, and the impact of propranolol and clonidine on this pathway is unknown. We hypothesized that seven days following severe trauma and chronic stress, rats

would have increased bone marrow expression of HGF, c-Met, and MMP-9 as well as increased plasma corticosterone levels, increased HPC mobilization that is associated with decreased hemoglobin levels. Further, we hypothesized that beta blockade with propranolol and sympathetic outflow inhibition with clonidine would decrease HGF, c-Met, MMP-9, corticosterone, and HPC mobilization, resolving persistent injury-associated anemia.

Methods

Animals

Male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing 300–400g were housed in pairs and fed *ad lib* with Teklad Diet #7912 (Harlan Laboratories Inc., Tampa, FL) and water during a one week acclimation period. Light and dark cycles were 12 hours each throughout acclimation and experimental periods. All animal care was conducted in accordance with University of Florida Institutional Animal Care and Use Committee standards. Animal injury models were performed as previously described, using isolated lung contusion to recapitulate blunt trauma, using lung contusion with hemorrhagic shock to recapitulate blunt trauma with life-threatening hemorrhage, and using lung contusion with hemorrhagic shock followed by chronic stress in a restraint cylinder to recapitulate blunt trauma with life-threatening hemorrhage followed by daily stressors or the ICU environment associated with hypercatecholaminemia, neuroendocrine activation, and bone marrow dysfunction manifest as anemia which is reversible by attenuating the impact of hypercatecholaminemia and neuroendocrine activation with propranolol or clonidine (12, 13). Animals were randomized to ten groups, as listed in Table 1. Animals were sacrificed on post-injury day seven by cardiac puncture following intraperitoneal injection of ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg). Bone marrow, peripheral blood, and plasma were collected.

Lung contusion

Prior to performing LC, animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). LC was performed by applying a percussive staple gun (PowerShot Model 5700M, Saddle Brook, NJ) to a 12 mm metal plate applied to the right lateral chest wall 1–2 cm below the axillary fold. This technique generates a clinically significant and reproducible pulmonary contusion, based on histologic findings (3, 14, 15).

Hemorrhagic shock

Immediately following LC, animals randomized to LCHS or LCHS/CS were placed on a heating pad to maintain normothermia, and PE-50 tubing was inserted into the right internal jugular vein and right femoral artery under direct visualization. The arterial catheter was applied to a BP-2 Digital Blood Pressure Monitor (Columbus Instruments, Columbus, OH) for continuous blood pressure monitoring. Blood was withdrawn through the venous catheter into a heparinized syringe until a mean arterial pressure of 30–35 mm Hg was reached. This blood pressure range was maintained for 45 minutes by withdrawing or reinfusing blood as needed. After 45 minutes, shed blood was reinfused at 1 mL/min. No additional resuscitation fluids were administered.

Chronic stress

Chronic restraint stress was incorporated to simulate stressors associated with the intensive care unit environment for human trauma patients. For animals randomized to LCHS/CS, CS began one day after LCHS, and was performed by placing animals in a restraint cylinder (Kent Scientific, Torrington, CT) for two hours each day until sacrifice on day seven. To prevent acclimation, the cylinders were rotated 180 degrees every 30 minutes, and alarms (80 dB) were transmitted by speakers adjacent to the restraint cylinders for two minutes each time the cylinders were rotated. Previous work demonstrates that these interventions induce neuroendocrine activation and inflammation consistent with chronic stress associated with severe injury and ICU admission for trauma patients (16, 17). All non-CS groups were subjected to a two hour daily fast while CS was being performed.

Propranolol and clonidine

Propranolol and clonidine were administered by intraperitoneal injection 10 minutes following resuscitation from hemorrhagic shock (i.e. systolic blood pressure 80 mm Hg), and then daily for six days following CS or daily handling. Propranolol and clonidine doses were 10 mg/kg and 75 µg/kg, respectively, based on previous work demonstrating the safety and efficacy of these doses in reducing heart rate by 10–20% without causing significant hypotension (14, 18). There were occasional mild decreases in systolic blood pressure (10 mm Hg) following propranolol or clonidine administration but no apparent cases of medication-induced shock following propranolol or clonidine administration.

Bone marrow HGF, c-Met, and MMP-9

Bone marrow HGF, c-Met, and MMP-9 were chosen as analytes because they have been implicated in the pathophysiology of stress-induced HPC mobilization (4–6, 9, 10). Bone marrow expression of HGF, c-Met, and MMP-9 were assessed by real-time polymerase chain reaction. The following primers were used: HGF: forward 5' tgcaacggtgaaagctacag, reverse 5' agcccttgctcgggatattt (product region: 785–908, product size: 124 base pairs), C-Met: forward 5' gaggaagagtcaggccagt, reverse 5' tcggagggttaggaagagtt (product region: 499–626, product size: 128 base pairs), MMP-9: forward 5' cactgtaactgggggcaact, reverse 5' agagtactgctgccagga (product region: 1008–1080, product size: 73 base pairs). Results were presented as mRNA fold change relative to naïve expression levels.

Plasma corticosterone

Plasma corticosterone was chosen as an analyte because it has been implicated in the pathophysiology of stress-induced HPC mobilization (11). Plasma corticosterone was measured by enzyme linked immunosorbent assay (Abcam, Cambridge, MA) performed according to manufacturer instructions with samples run in duplicate.

Hematopoietic progenitor mobilization

Mobilization of hematopoietic progenitor cells from the bone marrow to peripheral blood was assessed by flow cytometry. Blood was stained with CD71 antibodies (BD Biosciences, San Jose, CA) to identify hematopoietic progenitors, which are more differentiated than hematopoietic stem cells. (Southern Biotech, Birmingham, AL) conjugated to phycoerythrin

(BD Biosciences, San Jose, CA). Hematopoietic progenitors were identified as CD71+/c-kit+, analyzed by a BD LSR II flow cytometer with FACSDiva software (BD Biosciences, San Jose, CA).

Hemoglobin

Peripheral blood hemoglobin levels (g/dL) were assessed in heparinized whole blood samples by a hematology analyzer (Abaxis, Union City, CA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.05 (GraphPad Software, La Jolla, CA) to calculate one-way analysis of variance. Data were reported as mean $\pm$ standard deviation. Significance was set at $\alpha = 0.05$. In figures, a single asterisk (*) was used to denote $p < 0.05$ compared with naïve animals; two asterisks (**) were used to denote $p < 0.05$ compared with untreated counterparts who had the same injury but did not receive propranolol or clonidine.

Results

Bone marrow HGF, c-Met, and MMP-9

Bone marrow expression of HGF, c-Met, and MMP-9 is illustrated in Figure 1A–C. LC, LCHS, and LCHS/CS groups had increasing bone marrow expression of HGF, c-Met, MMP-9 that correlated with increasing injury severity, i.e. LC < LCHS < LCHS/CS. Compared to naïve animals, LCHS/CS animals had significantly higher bone marrow expression of HGF (2.3 ± 0.9 , $p < 0.001$) and MMP-9 (3.3 ± 2.7 , $p = 0.006$).

In the LCHS/CS group, bone marrow HGF was slightly reduced by propranolol (1.9 ± 0.7) and clonidine (1.6 ± 0.9), though the expression level remained significantly higher than naïve animals ($p < 0.001$ and $p = 0.019$, respectively), and was not significantly lower than untreated LCHS/CS animals ($p = 0.490$ and $p = 0.233$, respectively). However, MMP-9 was significantly reduced by propranolol (1.1 ± 0.4 , $p = 0.023$) and clonidine (1.5 ± 1.5 , $p = 0.035$) compared to untreated counterparts, and was not significantly greater than naïve ($p = 0.425$ and $p = 0.348$, respectively).

Plasma corticosterone

Plasma corticosterone levels are illustrated in Figure 2. Plasma corticosterone levels (ng/mL) increased with increasing injury severity in each of the rodent models (naïve: 115 ± 51 , LC: 161 ± 65 , LCHS: 182 ± 91 , LCHS/CS: 276 ± 148). Plasma corticosterone was significantly increased following LCHS/CS compared to naïve ($p = 0.043$).

Following LCHS/CS, plasma corticosterone levels were significantly decreased by the addition of propranolol (146 ± 53 , $p = 0.016$) and clonidine (151 ± 47 , $p = 0.041$).

Hematopoietic progenitor mobilization

Hematopoietic progenitor mobilization data are illustrated in Figure 3. Compared to naïve animals ($1.2 \pm 0.7\%$), HPC mobilization was slightly increased seven days following LC (1.8

± 1.0 , $p=0.140$), significantly higher following LCHS (2.7 ± 1.9 , $p=0.015$), and highest following LCHS/CS (5.4 ± 1.8 , $p<0.001$).

Following LCHS, HPC mobilization was decreased by the addition of propranolol (1.4 ± 0.8) and clonidine (1.8 ± 0.7), though the differences were not statistically significant ($p=0.093$ and $p=0.114$, respectively). Following LCHS/CS, HPC mobilization was significantly decreased by the addition of propranolol (2.2 ± 0.9 , $p<0.001$) and clonidine (1.7 ± 0.5 , $p<0.001$).

Hemoglobin

Hemoglobin levels (g/dl) seven days following LC alone were similar to naïve (14.4 ± 0.8 vs. 14.3 ± 0.4 , $p=0.706$) (Figure 4). Compared to naïve animals, hemoglobin was significantly decreased seven days following LCHS (13.4 ± 1.2 , $p=0.022$), and was lowest following LCHS/CS (12.3 ± 1.2 , $p<0.001$).

Following LCHS, hemoglobin was increased by the addition of propranolol (14.3 ± 0.9) and clonidine (13.8 ± 0.5), though propranolol and clonidine did not significantly increase hemoglobin compared to untreated counterparts ($p=0.070$ and $p=0.414$, respectively). Following LCHS/CS, our most clinically relevant rodent model, hemoglobin was significantly increased by the addition of propranolol (13.7 ± 0.4 , $p=0.004$) and clonidine (14.1 ± 1.1 , $p=0.001$) compared to untreated counterparts.

Discussion

Following blunt traumatic injury with hemostasis, HPCs mobilize from the bone marrow to the peripheral blood and to site of injury, encouraging wound healing and tissue repair (3). When blunt trauma is accompanied by hemorrhagic shock and chronic stress, HPC mobilization is prolonged, leaving the bone marrow devoid of progenitors that are necessary to restore erythrocyte mass, resulting in bone marrow dysfunction and anemia. Our results suggest that HGF, c-Met, MMP-9, and corticosterone may each play a role in mobilizing HPCs from the bone marrow to peripheral blood following severe traumatic injury and chronic stress, consistent with known interactions among HPC mobilization pathways, as illustrated in Figure 5. Attenuating the neuroendocrine stress response with propranolol and clonidine had the greatest effects on MMP-9 and corticosterone. HGF and c-Met may have been less affected because they are indirectly influenced by the neuroendocrine stress response via upstream modulation of G-CSF, whereas MMP-9 and corticosterone are directly stimulated by high catecholamine levels and pro-inflammatory cytokines (9, 10). Untreated animals had prolonged increases in HPC mobilization and anemia seven days after injury; propranolol and clonidine each decreased HPC mobilization and abrogated persistent injury-associated anemia (13).

Mobilization of HPCs by rodents subjected traumatic injury and hemorrhagic shock appears to be mediated by beta-2 and beta-3 adrenergic receptors in a process that is reversed by non-selective beta blockade, as demonstrated by Beiermeister et al. (2). Therefore, non-selective beta blockade with propranolol and sympathetic outflow inhibition with clonidine may inhibit HPC mobilization. This application of propranolol and clonidine is especially

germane in the setting of persistent activation of the neuroendocrine stress response, which has been achieved by performing daily restraint stress following traumatic injury and hemorrhagic shock, resulting in persistent elevation of urine norepinephrine levels (16). Beta blockade with propranolol has shown efficacy in decreasing plasma G-CSF levels seven days after the combination of injury, shock, and daily restraint stress, consistent with the hypothesis that the neuroendocrine stress response upregulates G-CSF (14). Previously, animals treated with propranolol had less HPC mobilization from the bone marrow to the peripheral blood but did not have histologic evidence of impaired wound healing (19, 20). In the same model of injury, shock, and daily restraint stress, clonidine has been shown to decrease plasma G-CSF levels and HPC mobilization to the peripheral blood (18). Therefore, modulating the neuroendocrine stress response to severe injury and chronic stress with propranolol and clonidine has the potential to attenuate HPC mobilization, bone marrow dysfunction, and anemia without adversely affecting wound healing and tissue repair. The clinical translation of these findings depends on effectively recapitulating neuroendocrine activation and inflammation experienced by severely injured trauma patients requiring ICU admission. Based on previous work, the authors believe that the experimental methods presented herein accomplish this goal (16, 17). However, future research should investigate the efficacy of other potentially useful methods for recapitulating chronic ICU stressors, such as dysregulation of day and night sleep-wake cycles, which were not used in the present study, representing a methodological limitation.

Although propranolol and clonidine act by different mechanisms, they had similar effects on HPC mobilization pathways following severe injury and chronic stress. Propranolol is a non-selective beta adrenergic receptor antagonist that blocks the MAPK, JNK, and NF- κ B stress response pathways occurring downstream of the NE-beta adrenergic receptor complex (21, 22). Clonidine blocks sympathetic nervous system outflow by acting as a central alpha-2 agonist, and has been shown to attenuate the inflammatory response to surgery and experimental sepsis (23, 24). Because both propranolol and clonidine were effective in decreasing HPC mobilization and abrogating persistent injury-associated anemia, it seems that central sympathetic blockade is effective but unnecessary. This observation may have important implications for clinical translation.

Although both propranolol and clonidine are used clinically, propranolol seems more apt for use in trauma patients, especially in the tenuous period immediately following initial resuscitation. Clonidine has a tendency to promote hypotension, and has effects lasting for 6–10 hours, whereas propranolol has been safely administered to severely injured trauma patients following resuscitation and stabilization within 24 hours of admission (12). In a pilot study, trauma patients receiving propranolol had decreased growth of cultured HPCs beginning one day after injury and persisted 14 days after injury, suggesting that propranolol may have decreased HPC mobilization among human trauma patients (12). In this study, beta-blockers were not administered until initial resuscitation was complete and serum lactate was 4 mg/dL or less, and was then titrated to a 10% to 20% decrease in heart rate. Under these conditions, propranolol was held for one patient due to bradycardia, and there were no other adverse effects.

Our study was limited by a lack of mechanistic detail. The effects of increasing injury severity, initial versus persistent sympathetic activation due to chronic stress, and different mechanisms of action for propranolol and clonidine allow for the identification of associations, but do not establish causal relationships. In addition, this study was limited by the exclusive use of male rats. Male rats were used to avoid the potentially confounding effects of female hormones on the physiologic response to hemorrhagic shock. However, there may be important differences between male and female rats in the pathophysiology of HPC mobilization following severe injury and chronic stress, and further research in this area is needed. In addition, future research should also investigate the impact of aging on HPC mobilization and persistent injury-associated anemia, given that norepinephrine levels rise with increasing age, and aging has been associated with reduced hematopoietic stem cell proliferative and regenerative capacity (25–31). Therefore, the elderly may be particularly vulnerable to post-injury HPC mobilization and persistent injury-associated anemia. Finally, HGF and c-Met may have been less affected because they are indirectly influenced by the neuroendocrine stress response via upstream modulation of G-CSF. This hypothesis should also be tested in future studies.

Conclusions

Severe injury was associated with increased bone marrow expression of HGF, c-Met, and MMP-9, increased circulating corticosterone, prolonged mobilization of HPCs to the peripheral blood, and persistent anemia. Attenuating the neuroendocrine response to injury and stress with propranolol and clonidine led to reduced bone marrow MMP-9 expression, decreased circulating corticosterone levels, reduced HPC mobilization, and resolution of anemia. Future studies should evaluate the safety and efficacy of propranolol in preventing prolonged HPC mobilization and persistent injury-associated anemia in human trauma patients.

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Figure 1: Bone marrow expression of HGF (Figure 2A), c-Met (Figure 2B), and MMP-9 (Figure 2C) following severe trauma and chronic stress with and without propranolol and clonidine. LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress. * $p < 0.05$ vs. Naïve, ** $p < 0.05$ vs. untreated counterpart.

Figure 2:

Plasma corticosterone levels were slightly elevated following severe trauma and chronic stress, and were closer to naive levels when propranolol and clonidine were administered.

LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress.

Figure 3:

The effects of propranolol and clonidine on persistent hematopoietic progenitor cell (HPC) mobilization from the bone marrow to the peripheral blood seven days after injury, measure by flow cytometry to detect CD 71+/c-kit+ cells. LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress. * $p < 0.05$ vs. Naive, ** $p < 0.05$ vs. untreated counterpart.

Figure 4:

The effects of propranolol and clonidine on hemoglobin seven days after injury. LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress. * $p < 0.05$ vs. Naïve, ** $p < 0.05$ vs. untreated counterpart.

Figure 5: Conceptual diagram of hematopoietic progenitor cell (HPC) mobilization from bone marrow to peripheral blood. The neuroendocrine stress response upregulates the granulocyte colony-stimulating factor (G-CSF)/stromal cell derived factor-1 (SDF-1) axis. SDF-1 interacts with the CXCR4 receptor on bone marrow leukocytes, producing hepatocyte growth factor (HGF), which interacts with the c-Met receptor on HPCs, promoting their egress from the bone marrow to peripheral blood. Neuroendocrine and G-CSF activation also upregulates matrix metalloproteinase-9 (MMP-9) production in bone marrow leukocytes and HPCs, increasing HPC mobilization. Finally, elevated plasma corticosterone promotes HPC egress

to the peripheral blood. Prolonged HPC mobilization leaves the bone marrow devoid of progenitors that are necessary to restore erythrocyte mass, resulting in anemia.

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Table 1:

Injury model groups.

Group	n	LC	HS	CS	Propranolol	Clonidine
Naïve	10	–	–	–	–	–
LC	7	+	–	–	–	–
LC + Propranolol	8	+	–	–	+	–
LC + Clonidine	4	+	–	–	–	+
LCHS	12	+	+	–	–	–
LCHS + Propranolol	8	+	+	–	+	–
LCHS + Clonidine	10	+	+	–	–	+
LCHSCS	11	+	+	+	–	–
LCHSCS + Propranolol	8	+	+	+	+	–
LCHSCS + Clonidine	8	+	+	+	–	+

LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress.