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Persistent injury-associated anemia and aging: novel insights

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Abstract

Background—Hypercatecholaminemia and bone marrow dysfunction have been implicated in the pathophysiology of persistent-injury associated anemia. The elderly may be more vulnerable to bone marrow dysfunction due to high basal and peak catecholamine levels and impaired hematopoietic progenitor growth. We hypothesized that aging would adversely affect persistent injury-associated anemia.

Methods—Male Sprague Dawley rats age 8-9 weeks and F344-BN rats age 25 months were randomized to: naïve controls, lung contusion plus hemorrhagic shock (LCHS), and LCHS plus daily chronic restraint stress (LCHS/CS). Urine norepinephrine was measured on days one and seven. Mobilization of hematopoietic progenitor cells (HPC), bone marrow colony forming units-erythroid (CFU-E) growth, and peripheral blood hemoglobin, mean corpuscular volume (MCV), and red cell distribution width (RDW) were assessed on day seven. **p < 0.05 young vs. aged counterpart by one-way analysis of variance.

Results—Aged rats had higher norepinephrine levels at naïve baseline (97** vs. 27 ng/mL) and seven days following LCHS/CS when compared to young (359** vs. 127 ng/mL). Following LCHS/CS, HPC mobilization was greater among young rats when compared to aged (5.4 vs. 2.5%). CFU-E growth was lower among aged animals for each group (naïve: 47** vs. 65; LCHS: 40** vs. 50; LCHS/CS: 38** vs. 44 cells/plate). Aged naïve rats had higher initial hemoglobin (15.2** vs. 14.3 g/dL), but lower MCV (48** vs. 59 fL/cell) and larger RDW at baseline, and greater differences seven days after LCHS/CS (MCV: 46** vs. 60 fL/cell; RDW: 17.4** vs. 16.3%).

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The authors have no relevant conflicts of interest.

Author contributions

TJL, KBK, CSC, SCB, CL, PAE, and AMM contributed to study design. TJL, KBK, CSC, JMP, and JCM contributed to data collection and analysis. All authors contributed to data interpretation and provided critical revisions to the manuscript.

Conclusions—Compared to young rats, aged rats had less HPC mobilization despite elevated basal and peak norepinephrine. Aged rats were disproportionately affected by impaired hematopoietic progenitor growth and an iron-restricted red blood cell phenotype at baseline which persisted seven days after injury. Further research is needed to assess how the clinical approach to persistent-injury-associated anemia should differ for elderly trauma patients.

Level of Evidence—Prognostic study, level III

Keywords

Anemia; erythropoiesis; aging; trauma; injury

Introduction

Trauma patients are often affected by anemia due to acute blood loss and high levels of circulating catecholamines and inflammatory cytokines, which suppress iron availability and inhibit erythropoiesis in the bone marrow, leading to persistent injury-associated anemia (1-5). The elderly may be especially vulnerable to this phenomenon due to baseline anemia, an exaggerated neuroendocrine stress response, and impaired bone marrow function.

Anemia affects approximately 10% of subjects age 65, and is associated with two-fold increased all-cause mortality among the elderly (6, 7). Basal plasma norepinephrine levels and peak levels in response to stress each rise with increasing age (8-11). Although bone marrow cellularity appears to be preserved among the elderly, aging has been associated with reduced hematopoietic stem cell proliferative and regenerative capacity (12-14).

Preclinical investigation of persistent injury-associated anemia has been performed using young (age 8-9 week) Sprague-Dawley rats (15-17). Like many strains, Sprague-Dawley rats are not suitable for aging studies because they are afflicted by age-dependent renal insufficiency, and typically do not survive to old age (18). In contrast, Fischer-Brown Norway (F344-BN) rats do not appear to be afflicted by age-dependent renal insufficiency, and are less affected by age-related cardiac and hematopoietic dysfunction than other commonly used rat strains (19-21). Importantly, under normal physiologic conditions, F344-BN rats maintain the capacity for erythropoiesis throughout their lifespan, with average hemoglobin 15.3 g/dL at 30 months of age (22). We chose to use 25 month-old F344-BN rats in these experiments, roughly corresponding to 65 year-old humans (23).

The purpose of this study was to compare persistent injury-associated anemia in young versus aged rats. We hypothesized that aging would be associated with an exaggerated post-injury catecholamine surge, decreased bone marrow growth of hematopoietic progenitor cells, and iron-restricted persistent anemia seven days after injury and daily restraint stress.

Methods

Animals

Male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing 300-400g and F344-BN rats age 25 months (generously donated by the National Institute of Aging) weighing 450-600g 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. Young and aged animals were randomly allocated to naïve (young: n=10, aged: n=8), lung contusion (LC) followed immediately by hemorrhagic shock (LCHS) (young: n=10, aged: n=8), and LCHS followed by daily restraint stress (LCHS/CS) (young: n=10, aged: n=8). Chronic restraint stress was incorporated into the injury models to simulate stressors associated with the intensive care unit environment. 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) on day seven.

Lung contusion

Animals randomized to LCHS or LCHS/CS 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 crease. This model has been shown to produce a clinically significant and reproducible pulmonary contusion based on histologic findings (24-26).

Hemorrhagic shock

Animals randomized to LCHS or LCHS/CS were placed on a heating pad, 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.

Chronic stress

For animals randomized to LCHS/CS, CS was performed by placing animals in a restraint cylinder (Kent Scientific, Torrington, CT) for two hours per day. To prevent acclimation, the cylinders were rotated 180 degrees every 30 minutes, and alarms (80 dB) were transmitted by speakers placed immediately adjacent to the cylinders for two minutes each time the cylinders were rotated. Because animals undergoing CS had no access to food or water in the restraint cylinder, all other groups were subjected to a two hour daily fast while CS was being performed.

Norepinephrine

Norepinephrine levels were measured in the urine as a surrogate for catecholamine levels in the blood because blood levels tend to be erratic with labile peaks and troughs, whereas urine norepinephrine is more stable over time (27, 28). Norepinephrine was assessed rather than epinephrine because previous work has demonstrated that post-injury anemia is primarily mediated by norepinephrine (29). On post-injury day one, spontaneous urine

samples were obtained during daily handling for non-CS groups, and were obtained during CS for CS groups. Spontaneous urine samples were also obtained on post-injury day seven prior to sacrifice. Samples were stored at -80°C . Urine norepinephrine was measured by enzyme linked immunosorbent assay (Labor Diagnostika Nord, Nordhorn, Germany).

Bone marrow cellularity

Bone marrow samples were obtained at the time of sacrifice by removing the left femoral epiphysis and flushing the femur with a 5 mL syringe containing Iscove's modified Dulbecco's Medium and 10% fetal bovine serum. The resulting suspension was passed through a 40 μm sterile nylon strainer, stained with 0.4% Trypan blue, and placed on a hemocytometer plate. Total viable cell counts were assessed by light microscopy.

Hematopoietic progenitor growth

Bone marrow cells were plated and incubated with a stock solution as previously described (30). Hematopoietic progenitor cell growth was assessed at three stages: colony forming unit – granulocyte, erythrocyte, monocyte, megakaryocyte (GEMM, early hematopoietic progenitors, counted fourteen day after plating), blast forming unit – erythroid (BFU-E, occurring between GEMM and CFU-E in the erythrocyte progenitor lineage, counted fourteen days after plating), and colony forming unit – erythroid (CFU-E, late hematopoietic progenitors, counted seven days after plating). Colony growth was assessed by light microscopy to identify colonies by morphology.

Hematopoietic progenitor mobilization

Mobilization of hematopoietic progenitor cells from the bone marrow to the peripheral blood was assessed by flow cytometry. Peripheral blood was stained with CD71 antibodies (BD Biosciences, San Jose, CA) and c-kit antibodies (Southern Biotech, Birmingham, AL) conjugated to phycoerythrin (BD Biosciences) to identify hematopoietic progenitors as CD71+/c-kit+. The percentage of hematopoietic progenitors in peripheral blood was calculated by a BD LSR II flow cytometer equipped with FACSDiva software (BD Biosciences).

Hemoglobin

Peripheral blood hemoglobin levels (g/dL) were measured on the day of sacrifice by analyzing heparinized whole blood samples in a hematology analyzer (Abaxis, Union City, CA).

Erythrocyte phenotype

Erythrocyte mean corpuscular volume (fL/cell) and red cell distribution width (%) were each measured on the day of sacrifice by analyzing heparinized whole blood samples in a hematology analyzer (Abaxis, Union City, CA). These parameters may be used to differentiate among different categories of anemia, e.g. iron restricted anemia is often manifest as reduced mean corpuscular volume with increased red cell distribution width.

Statistical analysis

Statistical analysis and figure production were performed using GraphPad Prism version 6.05 (GraphPad Software, La Jolla, CA) to calculate one-way analysis of variance for continuous variables. Significance was set at $\alpha = 0.05$ and data were reported as mean $\pm$ standard deviation.

Results

Norepinephrine

Urine norepinephrine levels are illustrated in Figure 1. Compared to young rats, aged rats had significantly higher urine norepinephrine levels (ng/mL) at baseline (97 ± 71 vs. 27 ± 32 , $p = 0.023$) and one day after LCHS (420 ± 239 vs. 23 ± 16 , $p = 0.007$). For aged and young rats, urine norepinephrine remained persistently elevated seven days after LCHS when animals were also subjected to CS (359 ± 99 vs. 127 ± 103 , $p = 0.004$), and aged animals had significantly greater norepinephrine levels under these conditions (359 ± 99 vs. 212 ± 130 , $p = 0.004$). Aged rats had higher urine norepinephrine than young rats seven days following LCHS as well, though the difference was not statistically significant (212 ± 130 vs. 61 ± 9 , $p = 0.090$).

Bone marrow cellularity

Bone marrow cellularity values are illustrated in Figure 2. At naïve baseline, aged animals had bone marrow cellularity (cells $\times 10^6$ /mL) similar to that of young naïve animals (231 ± 55 vs. 218 ± 46 , $p = 0.511$). Aged animals had bone marrow cellularity similar to that of young animals seven days after LCHS (181 ± 37 vs. 202 ± 40 , $p = 0.237$) and LCHS/CS (168 ± 38 vs. 189 ± 41 , $p = 0.224$). For aged and young animals, bone marrow cellularity after LCHS/CS was significantly lower than age-matched naïve animals.

Hematopoietic progenitor growth

Hematopoietic progenitor colony growth is illustrated in Figure 3. Compared to young animals, at naïve baseline, aged animals had decreased growth (colonies/plate) of GEMM colonies (23 ± 1 vs. 35 ± 4 , $p < 0.001$), BFU-E colonies (44 ± 4 vs. 56 ± 6 , $p < 0.001$), and CFU-E colonies (47 ± 4 vs. 65 ± 5 , $p < 0.001$). CFU-E growth, representing late hematopoietic progenitor growth, remained significantly lower among aged animals following LCHS (40 ± 1 vs. 50 ± 5 , $p < 0.001$) and LCHS/CS (38 ± 3 vs. 44 ± 5 , $p = 0.013$).

Hematopoietic progenitor mobilization

Hematopoietic progenitor mobilization results are illustrated in Figure 4. For both aged and young naïve animals, approximately 1.2% of all peripheral blood cells carried surface markers representing hematopoietic progenitors. Progenitor mobilization was similar between aged and young animals seven days after LCHS (2.2 ± 1.2 vs. 2.8 ± 1.9 , $p = 0.597$) and was significantly lower among aged animals seven days after LCHS/CS (2.5 ± 2.4 vs. 5.4 ± 1.8 , $p = 0.034$).

Hemoglobin

Hemoglobin levels are illustrated in Figure 5. Aged naïve animals had baseline hemoglobin levels that were significantly higher than young naïve animals (15.2 ± 0.9 vs. 14.3 ± 0.4 , $p = 0.023$), but not significantly higher seven days after LCHS (14.3 ± 1.0 vs. 13.4 ± 1.2 , $p = 0.096$) and LCHS/CS (13.3 ± 1.3 vs. 12.3 ± 1.2 , $p = 0.0667$). For aged and young animals, hemoglobin levels after LCHS/CS was significantly lower than age-matched naïve animals.

Erythrocyte phenotype

Erythrocyte phenotype parameters are illustrated in Figure 6. Aged naïve animals had lower erythrocyte mean corpuscular volume compared to young naïve animals (48 ± 3 vs. 59 ± 5 , $p < 0.001$), and this difference persisted levels seven days after LCHS (47 ± 1 vs. 59 ± 3 , $p < 0.001$) and LCHS/CS (46 ± 1 vs. 60 ± 4 , $p < 0.001$). Red cell distribution width was similar between aged and young animals at naïve baseline (17.0 ± 0.6 vs. 16.6 ± 1.3 , $p = 0.466$), but was significantly greater among aged animals seven days after LCHS (16.9 ± 0.3 vs. 16.2 ± 0.8 , $p = 0.038$) and LCHS/CS (17.4 ± 0.2 vs. 16.3 ± 1.1 , $p = 0.013$).

Discussion

Our results indicate that aged rats are disproportionately affected by post-injury hypercatecholaminemia and bone marrow dysfunction. At naïve baseline, aged animals had higher urine norepinephrine, decreased hematopoietic progenitor growth, and smaller erythrocytes, though bone marrow cellularity was preserved and hemoglobin levels were slightly higher. Seven days after severe trauma and chronic stress, aged animals had persistently higher urine norepinephrine, had proportionally greater losses in bone marrow cellularity, continued to have lower progenitor growth, and developed an iron-restricted anemia phenotype. Mobilization of hematopoietic progenitors from the bone marrow to peripheral blood affected young animals to a greater degree than aged animals. Therefore, although both groups had significantly lower hemoglobin levels seven days after severe trauma and chronic stress, the mechanisms contributing to persistent-injury associated anemia were different between aged and young animals.

The finding that aged animals had higher naïve basal and peak levels of urine norepinephrine than young counterparts is consistent with previous reports. It has been proposed that this phenomenon may be attributable to increased activity of dopamine- β -hydroxylase, the enzyme that synthesizes norepinephrine (31). In addition, norepinephrine end-organ metabolism may be subject to age-related changes. Uptake of norepinephrine into the heart is greater among aged rats than younger counterparts (32), though the inotropic response to norepinephrine is attenuated in aged animals (33). To our knowledge, the impact of aging on catecholamine levels following traumatic injury has not been previously reported, however, similar results have been reported in non-trauma stress. In a study comparing aged and young Fischer rats, McCarty et al. (10) found that one hour after cold water immersion, norepinephrine levels were significantly higher in the aged rats compared to young rats. In a study comparing elderly and young human subjects, Barnes et al. (11) found that psychologic stress-induced norepinephrine levels were significantly higher among the elderly subjects.

Total bone marrow cellularity among aged animals was preserved, and slightly increased, compared to young animals. This observation was consistent with that of Stelzer et al. (14), who found that the frequency of myeloid progenitor cells in the bone marrow was not significantly different between aged and young Sprague-Dawley rats. Notably, this study also found that aged rats had significantly reduced myeloid progenitor proliferative capacity, similar to our findings. These observations have also been made in human subjects. Human bone marrow cellularity and hematopoietic progenitor counts remain stable or slightly increased with advanced age (34-36). However, functional inhibition is suggested by the finding that both anemic and non-anemic elderly humans have reduced proliferation of CFU-E colonies (36, 37).

In this study, mobilization of hematopoietic progenitors from the bone marrow to peripheral blood was greater in young rats than aged rats. Unfortunately, there is a paucity of previously reported data regarding the effects of aging on hematopoietic progenitor mobilization. In a study of rhesus monkeys, Lee et al. (38) found that there was a greater proportion of circulating erythroid and myeloid progenitors among young animals compared to adult and elderly animals, consistent with our results. In humans, first trimester fetal blood contains high quantities of hematopoietic progenitors, and the quantity appears to decrease with increasing age (39, 40). Therefore, our observation that hematopoietic progenitor mobilization seven days following LCSH/CS was greater in young animals may be attributable to general aging pathophysiology rather than the unique pathophysiology of severe trauma and chronic stress.

Aged animals had significantly greater hemoglobin levels than young animals at naïve baseline. F44-BN animals have been previously shown to have increased hemoglobin levels later in their lifespan, with average hemoglobin 15.3 g/dL at 30 months of age, similar to our observation that 25 month old animals had hemoglobin 15.2 g/dL (22). However, this phenomenon does not occur in humans. The prevalence of anemia among human subjects increases with increasing age, whether considering community-dwelling subjects (6) or critically ill medical and surgical patients (41). It is likely that these trends are observed in trauma patients as well. In a propensity matched analysis comparing trauma patients age 65 or greater to trauma patients age 18-64, we have previously observed that admission hemoglobin levels are significantly lower among the elderly (10.2 ± 1.9 vs. 11.3 ± 2.0 g/dL, $p = 0.012$, unpublished data). In the present study, the significantly higher baseline hemoglobin level in aged animals was attenuated following LCHS and LCHS/CS, and both groups were significantly anemic seven days after LCHS/CS. The authors believe that injured animals exhibited a mild-moderate anemia rather than severe anemia because compensatory mechanisms to upregulate erythropoiesis were partially effective in mitigating post-injury anemia. In the clinical setting, bone marrow failure alone may not manifest as severe anemia requiring red cell transfusion, but it may contribute to severe anemia when considered in the context of acute and chronic blood loss among critically ill trauma patients.

Notably, aged animals had significantly lower mean corpuscular volume and slightly greater red cell distribution width, and these differences were exaggerated by severe trauma and chronic stress. These findings suggest the presence of iron-restricted erythropoiesis in the aged animals (42, 43). To our knowledge, the effects of trauma on erythrocyte phenotype in rats

have not been previously reported. In humans, the erythrocyte phenotype of anemic and non-anemic subjects varies according to the underlying disease process. In our study, F344-BN rats were used for the aged group because they are less affected by age-related degenerative disease (19-21). However, it is possible that they were affected by anemia of chronic disease, causing iron sequestration and an iron-restricted erythrocyte phenotype. Unfortunately, the use of heparin as an anticoagulant in our plasma samples limits our ability to investigate the iron-sequestration pathway in greater detail.

The major limitations of this study are the small number of animals (n=8-10 per group), the use of different rat strains to represent aged and young groups, and lack of mechanistic detail regarding iron-restricted anemia. Although the sample sizes were small, they were adequate to detect statistically significant differences between groups that were consistent with previously reported findings under different experimental conditions, suggesting that this study was adequately powered. Ideally, aged F344-BN rats would be compared to young F344-BN rats to perform these analyses. Unfortunately, young F344-BN rats were not available when these experiments were being performed. In addition, the young Sprague-Dawley trauma model has been well validated in previous studies (15, 44, 45), establishing a reliable foundation for comparison to aged animals. Future studies should investigate the impact of hematopoietic progenitor mobilization on wound healing and tissue repair and elaborate on the impact of aging on iron-restricted post-injury anemia by incorporating plasma and serum iron studies and analysis of bone marrow iron stores by histology. Because hematopoietic progenitor mobilization appears to be necessary for wound healing and tissue repair (17, 46), and aged animals had less mobilization than young animals in the present study, this phenomenon may have clinically important implications for severely injured elderly trauma patients. In particular, decreasing progenitor mobilization abrogating the neuroendocrine stress response with non-selective beta adrenergic receptor blockers may be ineffective among elderly trauma patients. Further investigation of the impact of aging on post-injury iron dysregulation may elucidate a role for modulating hepcidin to improve iron bioavailability.

Conclusions

Compared to young rats, aged rats were disproportionately affected by post-injury hypercatecholaminemia, impaired hematopoietic progenitor growth, and an iron-restricted red blood cell phenotype at baseline which persisted seven days after injury. Although both groups developed significantly lower hemoglobin levels seven days after severe trauma and chronic stress compared to age-matched naïve animals, the mechanisms contributing to persistent-injury associated anemia appear to be different between aged and young animals. Further research is needed to determine if the clinical approach to persistent injury-associated anemia should differ between elderly and young trauma patients.

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Figure 1.

Aged rats had significantly higher urine norepinephrine levels at baseline and one day after lung contusion and hemorrhagic shock (LCHS). For young and aged rats, urine norepinephrine remained persistently elevated seven days after LCHS when animals are also subjected to daily restraint stress (CS), and aged animals had significantly greater norepinephrine levels under these conditions (* $p < 0.05$ vs. age-matched Naïve, ** $p < 0.05$ Young vs. Aged).

Figure 2.

Young and aged rats each had significantly decreased bone marrow (BM) cellularity seven days following lung contusion, hemorrhagic shock, and daily restraint stress (LCHS/CS, * $p < 0.05$ vs. age-matched Naïve).

Figure 3.

Aged naïve animals had decreased growth of granulocyte, erythrocyte, monocyte, megakaryocyte (GEMM) (A), blast forming unit – erythroid (BFU-E) (B), and colony forming unit – erythroid (CFU-E) (C) colonies compared to young naïve animals. These differences persisted seven days after lung contusion and hemorrhagic shock (LCHS) and LCHS followed by daily restraint stress (LCHS/CS, * $p < 0.05$ vs. age-matched Naïve, ** $p < 0.05$ Young vs. Aged).

Figure 4.

In both young and aged naïve animals, approximately 1-2% of peripheral blood (PB) carried hematopoietic progenitor surface markers. Mobilization of hematopoietic progenitors from the bone marrow to the peripheral blood was significantly increase seven days after lung contusion and hemorrhagic shock (LCHS) and LCHS followed by daily restraint stress (LCHS/CS) for young animals, but not significantly increased among aged animals (* $p < 0.05$ vs. age-matched Naïve, ** $p < 0.05$ Young vs. Aged).

Figure 5.

Aged naïve animals had significantly higher baseline hemoglobin levels than young naïve animals, but similar hemoglobin levels seven days after lung contusion and hemorrhagic shock (LCHS) and LCHS followed by daily restraint stress (LCHS/CS). Young and aged animals each had significantly lower hemoglobin levels following LCHS/CS compared to age-matched naïve animals (* $p < 0.05$ vs. age-matched Naïve, ** $p < 0.05$ Young vs. Aged).

Figure 6.

Aged naïve animals had lower erythrocyte mean corpuscular volume (MCV) compared to young naïve animals, and this difference persisted levels seven days after lung contusion and hemorrhagic shock (LCHS) and LCHS followed by daily restraint stress (LCHS/CS) (A). Red cell distribution width (RDW) was similar between young and aged animals at baseline, but was significantly greater among aged animals seven days after LCHS and LCHS/CS (B) (* $p < 0.05$ vs. age-matched Naïve, ** $p < 0.05$ Young vs. Aged).