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Changes in the frequencies of human hematopoietic stem and progenitor cells with age and site

TL Farrell^a, TR McGuire^b, L Bilek^c, SK Brusnahan^d, JD Jackson^e, JT Lane^f, KL Garvin^g, BJ O'Kane^h, AM Bergerⁱ, SR Tuljapurkar^a, MA Kessinger^j, and JG Sharp^a

^aDepartment of Genetics, Cell Biology & Anatomy, University of Nebraska Medical Center, 986395 Nebraska Medical Center, Omaha, NE 68198-6395, USA

^bPharmacy Practice, University of Nebraska Medical Center, Omaha, NE, USA

^cSchool of Allied Health Professions, University of Nebraska Medical Center, Omaha, NE, USA

^dPharmaceutical Sciences, University of Nebraska Medical Center, Omaha, NE, USA

^eInstitute for Regenerative Medicine, Wake Forest University, Winston-Salem, NC, USA

^fPulmonary, Critical Care and Sleep Medicine, University of Nebraska Medical Center, Omaha, NE, USA

^gOrthopaedic Surgery and Rehabilitation, University of Nebraska Medical Center, Omaha, NE, USA

^hSchool of Dentistry, Creighton University, Omaha, NE, USA

ⁱAdult Health and Illness, College of Nursing, University of Nebraska Medical Center, Omaha, NE, USA

^jInternal Medicine Oncology/Hematology, University of Nebraska Medical Center, Omaha, NE, USA

Abstract

This study enumerated CD45hi/CD34+ and CD45hi/CD133+ human hematopoietic stem cells (HSC) and granulocyte-monocyte colony forming (GM-CFC) progenitor cells in blood and trochanteric and femoral bone marrow in 233 individuals. Stem cell frequencies were determined by multi-parameter flow cytometry employing an internal control to determine the intrinsic variance of the assays. Progenitor cell frequency was determined using a standard colony assay technique. The frequency of outliers from undetermined methodological causes was highest for blood but less than 5% for all values.

The frequency of CD45hi/CD133+ cells correlated highly with the frequency of CD45hi/CD34+ cells in trochanteric and femoral bone marrow. The frequency of these HSC populations in trochanteric and femoral bone marrow rose significantly with age. In contrast, there was no significant trend of either of these cell populations with age in the blood. Trochanteric marrow GM-CFC progenitor cells showed no significant trends with age, but femoral marrow GM-CFC

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Address for Correspondence: J. Graham Sharp, Ph.D., Department of Genetics, Cell Biology & Anatomy, University of Nebraska Medical Center, 986395 Nebraska Medical Center, Omaha, NE 68105-6395, jsharp@unmc.edu, 402-559-4390.

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trended downward with age, potentially because of the reported conversion of red marrow at this site to fat with age.

Hematopoietic stem and progenitor cells exhibited changes in frequencies with age that differed between blood and bone marrow. We previously reported that side population (SP) multipotential HSC, that include the precursors of CD45hi/CD133+ and CD45hi/CD34+, decline with age. Potentially the increases in stem cell frequencies in the intermediate compartment between SP and GM progenitor cells observed in this study represent a compensatory increase for the loss of more potent members of the HSC hierarchy.

Keywords

Hematopoietic Stem Cells; Aging; Progenitor Cells

Introduction

In man, steady state hematopoiesis is generally successfully maintained with age ^{1,2}, but compensatory responses to stresses such as bleeding or cancer chemotherapy can be compromised ³, and stem cells are implicated ^{4,5}. Because of this situation, and also due to increased co-morbidities, chemotherapy doses employed for the elderly in general oncology are often reduced, and unfortunately, this can compromise therapy outcomes ^{6,7}. These data, along with increased risks of anemias, hematological diseases relating to myelodysplasias and malignancies, suggest that the human HSC compartment may be compromised with aging, similar to the mouse ⁸.

The effects of aging on the HSC compartment have been extensively studied in the mouse but, until recently, less so in man ^{8,9}. In the mouse, the effects of aging are strain dependent and the number of proliferating HSC has been related to lifespan ^{8,10}. In most mouse strains, HSC numbers decline with age, but in the long-lived C57Bl mouse, HSC numbers, especially side population (SP) HSC numbers increase and these SP HSC in older mice have an increased expression of anti-apoptotic genes ^{8,11}. In addition to changes in numbers, the differentiation potential of HSC shifts towards myeloid cells (myeloid bias) ⁹ and away from B lymphoid cells, in mouse and man ¹². The make-up of the T cell pool also shifts with a significant decrease in numbers and functions of new thymic emigrant cells ¹³.

It has been shown that the functions of HSC are compromised by aging ¹⁴⁻¹⁶ due to cellular senescence ^{17,18}, impaired DNA repair ¹⁹⁻²², or telomere erosion ^{23,24}. Although the consequences of this in man are uncertain ²⁵, this likely influences physical abilities ²⁶ and marrow failure ²⁷. Homing and engraftment of murine HSC is less efficient with aging ^{2,28}, and decreases in mitochondrial function with aging have been described ²⁹. In addition, it has been increasingly recognized that the HSC compartment exhibits heterogeneity ^{12,30} and that the mosaic of functional clones changes with aging ^{12,31} and, potentially also differs between individuals. Overall, this would account for variations in HSC numbers and functions with aging and, potentially, in sensitivities to chemotherapy.

Recent studies of changes in the frequency of human hematopoietic stem cells with aging are not all comparable potentially because different markers, e.g. SP versus lineage negative CD34+ or CD133+ cells (e.g. ³² versus ⁹) have been employed to identify HSC populations. Transplantation of these HSC populations to immunodeficient mice shows that their ability to produce circulating human mononuclear cells varies ³⁰. There are differing potentialities even within the HSC hierarchy, with SP cells having greater potential than CD34+ cells which, in turn, have greater potential than CD34- cells to produce these cells ³⁰. SP HSC numbers decline with age, although the surviving cells are of high quality ³². In contrast,

aldehyde dehydrogenase high, CD34+ and CD133+ human HSC in bone marrow have been reported to show no age-related declines although circulating progenitors did decline³³. However, several other studies showed increases in bone marrow CD34+ and CD133+ HSC numbers in older subjects^{9,34-36} indicating that additional data are needed to settle this question.

In 2003, we hypothesized at a National Institutes of Aging Workshop on stem cells, that the more primitive, undifferentiated and likely quiescent SP HSC, might be lost, or used up, during the aging process. This loss could be compensated by an increase in more differentiated HSC and more of these cells are likely proliferating and sensitive to chemotherapy. Hence, this might explain the increase in sensitivity of the elderly to chemotherapeutic agents. This hypothesis predicted that SP HSC cell numbers would decrease with aging³² but there would be a compensatory increase in CD34+ and CD133+ stem cells in the HSC compartment. Whether these changes would be evident only in bone marrow, or also observed in blood, was unclear because the relationship between these two HSC compartments is dynamic and its regulation uncertain and the role of the microenvironment in HSC aging is unclear^{16,37-39}, although age-related changes in stem cell niches in *Drosophila* testis cause a decline in germ cell self-renewal⁴⁰.

To test the hypothesis, 233 human subjects, of ages between 21 and 88 years, undergoing hip replacement surgery were enrolled in an IRB approved study which enumerated the SP HSC, CD34+ and CD133+ HSC by flow cytometry and myeloid colony forming cells (GM-CFC) in culture from the bone marrow of the trochanteric region of the femoral diaphysis and femoral head as well as in blood.

The results of study of SP HSC, which showed a decline in numbers with age but an increase in “quality” of the surviving cells, have been described previously³². This report presents the data on the changes in numbers of the intermediate compartment of CD34+ and CD133+ stem cells and progenitor cells, assayed as myeloid colony forming cells (GM-CFC) cells with age and with sites in bone marrow and blood as well as the correlations between these cell populations and aging. The results indicated differences in the frequencies of different HSC cell populations as well as differential changes based on the site of origin of HSC (blood versus trochanter marrow versus femoral head marrow) with age.

Materials and Methods

Human Subjects

The National Institute of Aging supported this study describing the relationship of stem cell numbers and quality to age and health status but had no role in data analysis or interpretation. Institutional Review Board approval was received to consent and enroll up to 240 individuals undergoing total hip replacement. Exclusion criteria included a diagnosis of avascular necrosis, any abnormal bone marrow condition, a history of malignancy, or any previous chemotherapy or radiation therapy. Peripheral blood samples, along with bone marrow from both the femoral head and trochanteric region, were collected from each subject at the time of surgery and processed within six hours. A detailed description of the technique employed to collect femoral and trochanteric bone marrow samples has been described previously^{32,41}.

Samples

Peripheral blood mononuclear cells (PBMC) were obtained using lymphocyte separation medium (Mediatech Inc., Manassas, VA 20109). Cells were harvested from femoral head

and trochanter bone marrow samples by gently crushing the bone using a mortar and pestle and washing with HBSS without Ca or Mg (Invitrogen, Carlsbad, CA 92008), containing 20% Fetal Bovine Serum (FBS; Hyclone, Logan, UT 84321), 13.5 µg/ml DNase (Sigma-Aldrich, St. Louis, MO 63178), and 10 U/ml sodium heparin (Elkins-Sinn Inc., Cherry Hill, NJ 08003). Mononuclear cells (MNC) from the trochanter and femoral head bone marrow mixture were harvested employing a density gradient. Each sample was digitally photographed and the depth of the supernatant fat layer measured along with the total depth of the samples. This allowed calculation of the amount of fat (mm/g) in the sample which was plotted against the age of the subject.

Determination of CD45hi/CD133+, CD45hi/CD34+ and in blood and bone marrow

One million PBMC and bone marrow samples (MNC) were stained with fluorochrome conjugated antibodies CD45-FITC plus CD133-PE or CD34-PE using standard phenotyping techniques (15 minutes, 4°C). Analysis was performed on a FACS Aria (Becton-Dickinson, San Jose, CA 95131); results were evaluated with FlowJo software (Tree Star, Ashland OR). Hematopoietic stem cells were defined and identified as CD45hi/CD133+ and CD45hi/CD34+ and reported as % PBMC (peripheral blood samples) or % MNC (bone marrow samples).

The numbers of GM-CFC in the collections were determined using a previously described culture technique³⁰. Briefly, 3×10^5 MNCs isolated from the bone marrow samples were plated in 35-mm Petri dishes containing Iscove's modified Dulbecco's medium, 20% fetal bovine serum, 50 µM 2-mercaptoethanol, 100 µg/mL streptomycin, 100 U/mL penicillin, and 0.3% agar supplemented with 200 U/mL interleukin-3, 200 U/mL GM-CSF, and 200 U/mL G-CSF. Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO₂ in air for 14 days at which time the plates were stained with Wright's Giemsa and colony numbers enumerated. Colonies were defined as groups of ≥ 30 cells.

Data and Statistical Analysis

A small number of assays failed quality control requirements, so there were minor variations in the number of subjects in various comparisons. An internal control was included to quantify technically derived variance. The CD34 antibody was included in two separate tubes (Samples A and B), and these results were compared for equivalency and the frequency of outliers (discrepancies of $\pm 20\%$) noted. A scatter plot was produced to look at the association of the cell count for each cell population (CD34+, CD133+ and GM-CFC) and age for trochanteric and femoral bone marrow and peripheral blood. Spearman Rank Correlation and linear regression were used to test for significant trends between various stem/progenitor cell phenotypes, age, and anatomic sites (blood, trochanter, and femur). In addition, prior data indicated that CD133+ cells are a subpopulation of CD34+ cells, so the frequency of both of these populations was compared based on the slope of the relationship for trochanteric and femoral bone marrow and peripheral blood. Based on pre-determined criteria, comparisons were considered significant at $p \leq 0.05$ and a trend at $p \leq 0.1$.

Results

Variations between cell populations and sites

Independent staining of CD45/CD34 in two separate tubes (Samples A and B, Figure 1) was employed to determine the inherent variance of the staining and analysis procedures. The plot of CD34+ cells in two separate tubes (Samples A & B) showed a straight line at slightly less than a 45° angle with a statistically significant P value of <0.0001 . This indicated that, methodologically, the data were internally consistent with generally less than 5% of values presenting as significant high or low outliers. Blood had the highest proportion of high and

low outliers. CD45hi/CD34+ and CD45hi/CD133+ cell populations (Figure 2) were highly correlated in peripheral blood and trochanteric and femoral head bone marrow samples ($p < 0.0001$) (Figure 2). However, the relative proportion of CD45hi/CD133+ cells in peripheral blood compared to CD45hi/CD34+ cells (33%) was lower than in trochanteric and femoral bone marrow (about $55 \pm 5\%$). These data suggest site specific variations in the relationships of CD45hi/CD133+ to CD45hi/CD34+ cells. Similarly, trochanteric marrow GM-CFC numbers correlated highly with femoral GM-CFC numbers (Figure 1D) although slightly more (by about 10%) GM-CFC per nucleated cell were observed in femoral bone marrow.

Variations with subject age and sites

In peripheral blood CD45hi/CD34+ cell populations showed no significant trend with age ($p = 0.57$) whereas, in contrast, CD45hi/CD133+ cell populations increased slightly with age ($p = 0.026$) (Figure 3A, Figure 3D).

In trochanteric bone marrow, both CD45hi/CD34+ and CD45hi/CD133+ cell populations increased significantly with age ($p = 0.0003$ and $p < 0.0001$ respectively) (Figure 3B, Figure 3E). CD45hi/CD34+ and CD45hi/CD133+ populations also increased significantly with age ($p = 0.0002$ and $p < 0.0001$, respectively) in femoral head bone marrow (Figure 3C, Figure 3F).

Although the trochanteric bone marrow GM-CFC numbers correlated highly with femoral bone marrow GM-CFC numbers (Figure 1D), trochanteric bone marrow GM-CFC numbers did not change with age (Figure 4A) whereas femoral bone marrow GM-CFC declined moderately, but significantly ($p = 0.047$) with age (Figure 4B). This potentially reflects the lower age at which femoral bone marrow converts from active red bone marrow inactive fatty bone marrow compared to trochanteric bone marrow⁴². Consequently, the number of femoral bone marrow GM-CFC was related to the amount of fat in the bone marrow (mm of fat per gram of marrow/tube) (Figure 4C). The number of femoral bone marrow GM-CFC declined as the amount of fat increased, however this decline did not attain statistical significance ($p = 0.067$). The relationship between fat and hematopoietic progenitor cell numbers was opposite to that of the GM-CFC. There was a positive relationship between fat and CD45hi/CD34+ cell numbers in the trochanter ($r = 0.16$, $p = 0.03$), however, no relationship was found in the femur. CD45hi/CD133+ cells in both the trochanter and femur demonstrated a positive trend with amount of bone marrow fat.

These data indicate that the frequency of HSC and progenitors with age varied for CD34, CD133 and GM-CFC cell populations as well as with sites of harvest. Differences were observed between CD34+ and CD133+ cells in blood with age and effects with age were less evident than in bone marrow. In bone marrow CD34+ and CD133+ HSC increased with age, which is compatible with the hypothesis and most, but not all, previous reports. Changes with age were less evident in the GM-CFC compartment.

Discussion

The internal controls of this experiment showed that the methods were valid. The data also confirms that the frequency of CD133+ and CD34+ cells correlated highly in blood and trochanteric and femoral bone marrow, but the ratio (CD133+/CD34+) varied with site, ranging from about 33% in blood to 55% in femoral marrow and trochanteric marrow, suggesting different dynamics of these cell populations at various sites with aging especially between blood and marrow. The basis of these differences is not known. The primary finding in this study was that the compartment of hematopoietic stem cells with intermediate potential, in this study defined as CD45hi/CD133+ and CD45hi/CD34+ cells, increased

significantly in trochanteric and femoral head bone marrow in humans with aging. Myeloid progenitor cells (GM-CFC) in femoral bone marrow declined somewhat but in trochanteric marrow showed little change with age, so this compensation shown by the intermediate compartment³² did not extend to the progenitor compartment. We have shown previously that high potential SP HSC in the blood and bone marrow decreased in frequency in humans with aging³². The results of the present analyses supports the hypothesis that the CD45hi/CD133+ and CD45hi/CD34+ populations exhibited a compensatory increase related to the decrease in the high potential multipotential SP HSC compartment of the stem cell population with aging. The increase in HSC was most obvious in bone marrow, but not significantly reflected in blood. Overall, the balance of other studies also favors an increase in the intermediate potential compartment of HSC in bone marrow with age. Since such cells likely have greater proliferative activity, this could explain why hematopoiesis in the elderly shows increased chemosensitivity. Furthermore, these observations are compatible with the description on the effects of inhibiting autophagy of high potential (SP) stem cells with aging⁴³. Autophagy is required for maintenance of HSC⁴⁴. Loss of autophagy in HSC leads to the expansion of progenitor cells in bone marrow⁴³ as observed in this study. In support of this postulate, administration of rapamycin, which promotes autophagy, counters the effects of aging and extends lifespan in mice⁴⁵.

In conclusion, we have shown that CD34+/CD133+ HSC increase with age in bone marrow from humans undergoing hip replacement surgery. Although the relative and absolute increase in CD34+ cells in human bone marrow has been reported previously³⁴, in contrast Povsic, et al.³³ found no relationship between progenitor cells in human bone marrow and aging. Additionally, the data indicate that the dynamics of stem cells in blood versus bone marrow are differentially regulated and not necessarily correlated, so that blood stem cell numbers cannot reliably be employed as surrogate for bone marrow HSC behaviors. The results of this study combined with prior studies suggests that likely quiescent HSC, with greater proliferative potential are used up with aging³² and there is a compensatory increase in intermediate potential HSC (CD34+ and/or CD133+). As noted above, this intermediate potential HSC population most probably includes a greater proportion of proliferating HSC. It is unclear as to whether more clones of HSC are involved in proliferative activities or whether the number of progeny produced by individual clones is increased (or both). The amplification of clones, or of clonal progeny, especially of cells that may have accumulated unrepaired or incompletely repaired DNA damage with aging, would also explain the increase in the frequency of myelodysplastic clones and malignancies with aging^{46,47}.

Potential mechanisms by which intermediate HSC numbers and/or proliferation increase with age are unclear. The levels of inflammatory cytokines increase with aging and these might stimulate stem cell production by feedback mechanisms. In addition, there is evidence of decreased blood flow with aging to at least some areas of HSC production, e.g. proximal femur and pelvis⁴². This might indicate local hypoxia is associated with increases in production of cytokines, potentially by mesenchymal cells that contribute to the stem cell niche and whose secretome is altered by hypoxia including increased production of hepatocyte growth factor and VEGF⁴⁸. Hypoxia has also been associated with increases in stem cell populations in breast cancer⁴⁹ and neural cells⁵⁰. Consequently, a plausible hypothesis to explain the observations of this study is that declines in blood flow, to at least some areas of bone marrow, results in local hypoxia which then promotes mesenchymal stem cell production of VEGF and other cytokines/chemokines that then amplify, proliferating intermediate potential HSC. This also might have relevance to the site specific alterations in HSC numbers with aging that were observed in this study. Confirmation of these potential interactions would offer the opportunity to intervene and modify the changes of HSC with aging, perhaps by promoting blood flow by increasing physical activity⁵¹ or by promoting autophagy. Alternatively, it may be possible to decrease the sensitivity of HSC

to radiation or chemotherapy, in cancer patients, many of whom are elderly, by altering relevant cytokine/chemokine levels in order to decrease the proportion of proliferating HSC^{52,53}.

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Figure 1.

Depiction of results evaluating quality of assay methods. A-C compares the number of CD45hi/CD34+ cells from 2 sample tubes (samples A & B) with the same specimen assayed simultaneously with the same procedure. (A = peripheral blood (PBMC), B = trochanteric bone marrow, C = femoral head bone marrow) The linear regression line is visually depicted and the Spearman correlation (r) noted. D compares results from GM-CFC assays of specimens from the same individual, but different locations, femoral vs. trochanteric bone marrow. Numbers represent colonies formed per 1×10^5 mononuclear cells. For all figures the linear regression line is visually depicted and the Spearman correlation (r) noted.

Figure 2.

Figure 2 compares the proportion of CD45hi/CD34+ cells with the less differentiated CD45hi/CD133+ cells. Cells are represented as % peripheral blood mononuclear cells (A) or % mononuclear cells from bone marrow. For all figures the linear regression line is visually depicted and the Spearman correlation (r) noted.

Figure 3.

Figure 3 demonstrates the effect of age on hematopoietic stem cells in the various compartments. Numbers of CD45hi/CD34+ (A) and CD45hi/CD133+ (D) in the peripheral blood are depicted as % peripheral blood mononuclear cells. HSC in the bone marrow (trochanteric - CD45hi/CD34+ (B) & CD45hi/CD133+ (E) and femoral - CD45hi/CD34+ (C) & CD45hi/CD133+ (F)) are depicted as % bone marrow mononuclear cells. For all figures the linear regression line is visually depicted and the Spearman correlation (r) noted.

Figure 4.

A & B results represent granulocyte myeloid colony forming cells (GM-CFC) colony number at day 14 per 1×10^5 bone marrow mononuclear cells plated (A = trochanter, B = femur) relative to age. To determine if fat infiltration in the femur affected colony formation, C represents a comparison between GM-CFC and the amount of fat harvested from the femur (mm fat/g bone marrow)