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Human blood and marrow side population stem cell and Stro-1 positive bone marrow stromal cell numbers decline with age, with an increase in quality of surviving stem cells: Correlation with cytokines

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Abstract

Hematological deficiencies increase with aging leading to anemias, reduced hematopoietic stress responses and myelodysplasias. This study tested the hypothesis that side population hematopoietic stem cells (SP-HSC) would decrease with aging, correlating with IGF-1 and IL-6 levels and increases in bone marrow fat. Marrow was obtained from the femoral head and trochanteric region of the femur at surgery for total hip replacement ($N = 100$). Whole trabecular marrow samples were ground in a sterile mortar and pestle and cellularity and fat content determined. Marrow and blood mononuclear cells were stained with Hoechst dye and the SP-HSC profiles acquired. Marrow stromal cells (MSC) were enumerated flow cytometrically employing the Stro-1 antibody, and clonally in the colony forming unit fibroblast (CFU-F) assay. Plasma levels of IGF-1 (ng/ml) and IL-6 (pg/ml) were measured by ELISA. SP-HSC in blood and bone marrow decreased with age but the quality of the surviving stem cells increased. MSC decreased non-significantly. IGF-1 levels (mean = 30.7, SEM = 2) decreased and IL-6 levels (mean = 4.4, SEM = 1) increased with age as did marrow fat (mean = 1.2 mm fat/g, SEM = 0.04). There were no significant correlations between cytokine levels or fat and SP-HSC numbers. Stem cells appear to be progressively lost with aging and only the highest quality stem cells survive.

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Keywords

Stem cells; Aging; IGF-1; IL-6; Marrow fat

1. Introduction

Increasingly it is evident that declines in body functions that are observed with aging, target tissues with stem cell populations (Rando, 2006; Sharpless and DePinho, 2007). The most detailed studies of the impact of aging on stem cell populations have evaluated hematopoietic stem cells (HSC) in mice (Morrison et al., 1996; de Haan and van Zant, 1999; Liang and van Zant, 2008; Pearce and Bonnet, 2009; Warren and Rossi, 2009). Most mouse strains, e.g. DBA mice, exhibit declines in HSC numbers and function with age (Kamminga et al., 2005). The C57Bl strain is an exception in that this long lived strain has an increase in SP-HSC with age (Pearce et al., 2007), albeit with decreased homing efficiency (Dykstra and de Haan, 2008). These mice also exhibit an increased proportion of lower side population stem cells (LSP) based on Hoechst 33342 vital dye exclusion (Pearce et al., 2007). The SP-HSC that survive with aging have decreased expression of apoptosis related and increased expression of survival genes (e.g. bcl2) suggesting potentially up-regulation of an export pump with age and survival of the “fittest” stem cells (Pearce et al., 2007). Liang and van Zant (2003) have related organismal survival to maintenance of stem cells with aging.

In humans, hematological deficiencies become increasingly evident with aging. These include anemias (Guralnik et al., 2005) associated with a decrease in the erythroid precursor compartment (Lipschitz et al., 1984). A more general decline in hematopoietic reserve capacity in the elderly was described (Lipschitz and Udupa, 1986). Baldwin (1988) supported this conclusion, but with the caution that comorbidities significantly influence the hematopoietic status of the elderly (Lipschitz, 1995). Steady state hematopoiesis was adequate in the majority of the elderly, but the ability to respond to stress such as chemotherapy was compromised (Chatta et al., 1994; Chatta and Dale, 1996; Balducci, 2001). Myelodysplasias are increased in elderly individuals (Keating, 1996; Jackson and Taylor, 2002; Rothstein, 2003). A potential underlying explanation for these diverse problems is that the number and/or quality of HSC are compromised by the aging process, although changes in the microenvironment could also play a role (Aul et al., 1995).

Until recently, extending studies in the mouse to man was difficult due to the lack of suitable non-transplant assays of stem cell quality. In 1996, Goodell et al. described the side population (SP) assay. She showed that all of the hematopoietic stem cell (HSC) activity in bone marrow was contained in a population of cells distinguishable by flow cytometry based on their ability to pump out, via an ABCG2 transporter (Kim et al., 2002) Hoechst 33342 dye. Bunting (2002) has suggested that this phenotype might be common to all stem cell populations. Furthermore, Robinson et al. (2005) have confirmed that the lower SP-HSC cells (LSP) outcompete the upper SP-HSC cells (USP) and whole bone marrow for long-term growth in culture and long-term repopulation *in vivo*. Consequently, enumeration of the SP population measures the total hematopoietic stem cell population of the marrow and the ratio of LSP:USP expresses the “quality” of the HSC population.

Currently, the most accepted assays of bone marrow stromal cell precursors of the hematopoietic microenvironment are the enumeration of clonal colony forming units fibroblast (CFU-F) (Friedenstein et al., 1981; Delorme and Charbord, 2007; Zannettino et al., 2007) and flow cytometric evaluation of Stro-1 positive cells (Simmons and Torok-Storb, 1991; Xu et al., 2009). These assays were employed in this study. The other

component of bone marrow that is known to change with age (increase) is bone marrow fat (Tavassoli, 1989). Hematopoiesis goes into regression with aging, in that the more peripherally located marrow converts to adipose tissue and is no longer involved in active hematopoiesis (Neumann, 1882). Any declines in hematopoiesis with advancing age in man could potentially reflect this increase in bone marrow fat content (Tavassoli, 1989).

Although cytokine levels change with aging (Takasaki et al., 1997), the response of CD34⁺ hematopoietic stem/progenitor cells to cytokines in culture was preserved (Bagnara et al., 2000). Changes observed in cytokine levels, especially IGF-1 and IL-6, have been implicated as having effects on hematopoiesis (Maeda et al., 2005; Garrett and Emerson, 2008). It has not been determined if changes in stem cells with aging correlates with changes in levels of these cytokines. Consequently, this investigation was devised to determine if SP-HSC numbers and quality were altered with aging and if any changes were associated with changes in microenvironmental stromal cells and bone marrow fat or altered cytokine levels.

2. Materials and methods

2.1. Human subjects

The National Institute of Aging supported this study describing the relationship of stem cell numbers and quality to age and health status. Institutional Review Board approval was received to enroll and follow individuals undergoing total hip replacement, following informed consent. This report reflects an analysis of data from the first 100 subjects. 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.

2.2. Samples

Blood was centrifuged ($1000 \times g$) for 10 min and plasma collected, aliquoted, and stored at -80°C for batched cytokine analysis using a sandwiched type ELISA (R&D Systems, Minneapolis, MN). Peripheral blood mononuclear cells were obtained by standard density gradient using lymphocyte separation medium (Mediatech Inc., Manassas, VA 20109). Bone marrow samples were pulverized in a mortar and pestle using HBSS (Invitrogen, Carlsbad, CA 92008) without Ca or Mg, containing 20% FBS (Hyclone, Logan, UT 84321), 13.5 $\mu\text{g}/\text{ml}$ DNase (Sigma-Aldrich, St. Louis, MO 63178), and 10 U/ml sodium heparin (Elkins-Sinn Inc., Cherry Hill, NJ 08003).

2.3. Determination of SP-HSC in blood and bone marrow

Cells were stained with the DNA binding Hoechst 33342 dye as described by Goodell et al. (1996) with minor modifications. Analysis was performed by flow cytometry using a dual laser FACS Aria (Becton-Dickinson, San Jose, CA). The Hoechst-33342 stained cells were excited at 354 nm (UV) and emission fluorescence measured at 675 nm (red, 675/20 bp filter) and 440 nm (blue, 440/40 bp filter) wavelengths. Emissions were separated using a 505 nm long pass dichroic mirror and data analyzed on linear axes. Hematopoietic stem cells, but not mesenchymal stem cells (MSC) were included in the SP cell profile.

2.4. Interleukin-6

100 μl of plasma was analyzed for IL-6 concentrations. The dynamic range of the assay was between 0.7 pg/ml and 300 pg/ml. Concentrations of IL-6 that exceeded the upper limit of the assay were diluted 1:4 and re-analyzed. The lower limit of sensitivity was 0.7 pg/ml and inter-assay and intra-assay coefficient of variation was less than 5%.

2.5. Insulin-like growth factor-1 (IGF-1)

20 µl of plasma was diluted 1:100 with acidified buffer to allow dissociation of IGF-1 from plasma proteins. The dynamic range of the assay was between 0.025 ng/ml and 6 ng/ml with inter-assay and intra-assay coefficient of variation below 10%. Concentrations of IGF-1 in plasma was calculated from the standard curve and multiplied by 100 to account for the 100:1 dilution.

2.6. Marrow stromal cell (MSC) measurement

2.6.1. CFU-F assay—Triplicate aliquots of 5×10^5 unstained and unsorted mononuclear cells, harvested from both the trochanteric region and the femoral head, were seeded in 35 mm tissue culture plates containing 2.0 ml of RPMI (Invitrogen) + 12.5% FBS and 12.5% HS (Invitrogen). Cultures were incubated at 37 °C in a 5% CO₂ content, humidified chamber. At 10 days, medium was discarded and plates were stained with Wright-Giemsa for 2 min, rinsed with tap water and air dried. An aggregate of ≥ 20 cells were classified as a colony. Additionally, Stro-1 positive bone marrow cells were enumerated. This analysis employed antibody produced from the Stro-1 hybridoma obtained from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa, expanded and labeled with APC (PhycoLink kit, PROzyme, San Leandro, CA) in the monoclonal Antibody Core Facility at UNMC. An antibody cocktail of CD45FITC/Stro-1APC was used to stain 10^6 bone marrow cells which were analyzed on a FACSaria (Becton-Dickinson, San Jose, CA). Unstained cells were used to establish the negative population for each antibody. Cells that expressed positive Stro-1 staining in conjunction with low intensity CD45 expression were considered to be MSC.

2.7. Data and statistical analysis

For presentation, the subjects were placed into age cohorts of less than 50 years, between 50 and 59 years, 60 and 69 years and 70 years and older since this resulted in approximately similar numbers of subjects per cohort. A small number of assays failed quality control requirements so minor variations in the number of subjects per assay occurred. The variations in these cohorts with age were tested statistically for significance ($P \leq 0.05$) employing Kruskal–Wallis, with Dunn’s Multiple Comparison Testing, Mann–Whitney Rank Sum Test or by least squares linear regression analysis of the trends in individual values with age. The slopes of these regression analyses were tested using a *t* test for significant differences from zero slope as calculated using the following formula:, with *t* having *n* – 2 degrees of freedom and *r* representing the correlation coefficient of the regression line (Mather, 1965). Significant differences are noted in the figures.

3. Results

The number of SP-HSC declined progressively with age, although, as in the mouse, this decline was not dramatic (van Zant and Liang, 2003). There was a steady decline in the number of bone marrow SP-HSC with increasing age of participants (Fig. 1). This decline was also evident for blood SP-HSC in the older cohort (Fig. 2). As SP-HSC stem cell numbers declined, there was a significant increase in the LSP:USP ratio (Fig. 3). Since LSP outcompete USP for long-term repopulation of lethally irradiated mice (Robinson et al., 2005), this finding suggests that the SP-HSC that survived in the older cohort are of high quality. This is similar to the situation in C57Bl mice (Pearce et al., 2007).

The number of clonogenic stromal cells in bone marrow trended lower with age but did not achieve statistical significance (Fig. 4). The number of Stro-1+ cells in blood showed considerable variance and no trends with age (data not shown). The content of fat in the

bone marrow increased progressively with age (Fig. 5). This is as expected (Tavassoli, 1989) and is similar to recent results reported for mice (Naveiras et al., 2009).

The levels of IGF-1 in the plasma of the subjects decreased progressively and significantly with aging (Fig. 6). In contrast, plasma IL-6 levels increased progressively and significantly with age (Fig. 7). These changes in cytokine levels with aging are as predicted based on prior studies (Mysliwska et al., 1998; Forsey et al., 2003; Johnson, 2006; Maggio et al., 2006; Glatt et al., 2007).

The individual values for SP-HSC numbers versus bone marrow stromal cells, fat, as well as IGF-1 and IL-6 levels for each subject were analyzed by correlation analysis. None of these correlations achieved statistical significance (data not shown).

4. Discussion

The primary finding of this study was that SP-HSC in the blood and bone marrow decreased in frequency in humans with aging. We have also observed this finding in most mouse strains, with the exception of C57Bl mice, as noted by others (Pearce and Bonnet, 2009). A recent review noted the lack of data concerning changes in hematopoietic stem cells in humans with aging and this study addresses this deficit in knowledge (Pearce and Bonnet, 2009). We found that the SP-HSC that survive with aging are of high quality, similar to the SP-HSC in old mice of the long-lived C57Bl strain (Pearce et al., 2007). This is compatible with the notion that stem cells that have accumulated nuclear or mitochondrial DNA damage may die by apoptosis or senescence, leaving only high quality stem cells as survivors. It is puzzling why these survivors do not repopulate the compartment unless niche functions are also compromised or there are inhibitors of stem cells in the elderly.

This study also found that MSC showed a non-significant decrease in numbers and clonality with aging and unexpectedly there were no significant correlations between CFU-F and Stro-1 positive cells. Although the decline in stem cell numbers with aging appears to relate more to intrinsic factors, than to declines in the hematopoietic microenvironment, it is possible that although numerically, stromal cells declined minimally, their functions could be compromised.

As indicated by prior studies (Tavassoli, 1989; Naveiras et al., 2009) the fat content of the marrow increased with age. This reduced the extent of hematopoiesis and could account for the decreased ability of older adults to respond to hematopoietic stresses (Chatta et al., 1994; Chatta and Dale, 1996; Balducci, 2001). Since there were similar changes in several parameters, e.g. increased IL-6 and marrow fat or decreased SP-HSC and IGF-1 levels, there may be an underlying factor that influences all of these parameters. The cause of the increased marrow fat content with age is unknown (Tavassoli, 1989). A companion evaluation was performed in cadavers without hip disease to evaluate if the living subjects were atypical. No differences were observed that might indicate that hip disease was a factor in outcomes. Since the bone marrow was sampled from the proximal femur (trochanteric region and femoral head) which is the site of entry of the blood vessels to this region, a role for the vasculature cannot be excluded. Perfusion studies have shown a decrease in blood flow to the proximal femur with aging (Lahtinen et al., 1981) and this is also associated with an increase in bone marrow fat and this, in turn, is linked to osteoporosis (Griffith et al., 2008).

As in most prior studies of aging, the IGF-1 levels in the plasma of our subjects decreased significantly and the levels of IL-6 increased significantly with aging. However, neither of these correlated directly with the changes in the number of SP-HSC. It was thought likely that the IL-6 levels might correlate with the marrow fat levels since this cytokine is one of

the adipokines. Surprisingly, this correlation, which was not statistically significant, had an obvious negative slope. Consequently, the source of increased levels of IL-6 in older adults is unclear.

In conclusion, this study shows a decline in SP-HSC in man with aging, albeit with the surviving cells being of high quality. The results are similar to observations in the mouse; depending on strain (Pearce and Bonnet, 2009), suggesting that the mouse is a valid model of human aging, provided several strains are evaluated. These declines in numbers of SP-HSC with aging might amplify the risks of clonal HSC abnormalities and account for some of the unexplained causes of anemia in the elderly.

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Fig. 1.
SP stem cells/100,000 bone marrow mononuclear cells (MNC), analyzed by flow cytometry, are plotted against age cohorts.

Fig. 2.
SP stem cells/100,000 peripheral blood mononuclear cells (MNC), analyzed by flow cytometry, are plotted against age cohorts.

Fig. 3.
The ratio of lower side population (LSP) to upper side population (USP), which is a measure of stem cells quality (see Robinson et al., 2005), is plotted against age cohorts.

Fig. 4. Fibroblast colony forming cells (FCFC) in bone marrow/ 5×10^5 MNC plated, representing clonal mesenchymal stromal cell precursors, are plotted against age cohorts.

Fig. 5.
The quantity of fat (mm/g specimen weight) in bone marrow samples is plotted versus age cohorts.

Fig. 6.
The levels (ng/ml) of plasma insulin-like growth factor 1 (IGF-1) as determined by ELISA plotted versus age cohorts.

Fig. 7.
The levels (pg/ml) of plasma interleukin 6 (IL-6) levels as determined by ELISA assay plotted against age cohorts.