Decreased IRF8 Expression in Aging Hematopoietic Progenitor/Stem Cells

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Authorship

D.L.S. oversaw the design, analyses, and writing of the manuscript. Y.E.C., E.L.P., and M.R.C. performed most of the experiments. N.E.S., F.R.A., J.P.R., and S.H. helped with the design, analyses, and interpretation of the studies as well as actively contributed to the writing of the manuscript. M.Y., E.B.L. and B.L.W. helped obtain the samples for the studies and actively contributed to the writing of the manuscript.

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Abstract

To determine how aging impacts gene expression in hematopoietic stem cells (HSCs), human CD34+ cells from bone marrow (BMCD34+) and mobilized stem cell products (PBCD34+38-) were examined using microarray-based expression profiling. The age-associated expression changes in CD34+ cells were then compared to age-associated expression changes in murine HSCs. Interferon regulatory factor 8 (IRF8) was the only gene with age-associated expression changes in all analyses, decreasing its expression in human CD34+ cells and murine HSCs. Microarray-based expression profiling found that IRF8 expression also decreased with aging in human T-cells, suggesting that the effects of aging on IRF8 expression may extend to more differentiated populations of hematopoietic cells. Quantitative-RT/PCR studies confirmed that IRF8 mRNA expression decreased with aging in additional samples of BMCD34+, PBCD34+38-, and T-cells, and IRF8 protein expression was found to decrease with aging and to correlate with mRNA levels in PBCD34+ cells. The results suggest that IRF8 may be a novel biomarker of aging for hematopoietic cells. Given that inactivation of IRF8 causes CML-like syndromes in mice and decreased IRF8 expression occurs in human hematopoietic malignancies, it will be critical to determine if decreased IRF8 expression plays a role in the increased incidence of hematopoietic malignancies in older adults.

Introduction

Hematopoietic stem cells (HSCs) from older mice display evidence of increased proliferation, cell cycle dysregulation, reduced repopulating potential, and increased myeloid skewing with differentiation.(1-3) In humans, the incidence of bone marrow failure and hematopoietic malignancies increases with aging,(4) leading one to speculate that the biological effects of aging in HSCs promote the development of hematopoietic diseases in older adults. Although recent studies have examined age-associated expression changes in long-term repopulating (LTR) murine HSCs,(5, 6) there are no studies examining the age-associated expression changes in similar populations of undifferentiated human cells. Therefore, we examined the age-associated expression changes in human CD34+, comparing the age-associated expression changes in human CD34+ cells to murine long-term repopulating (LTR) HSCs. These studies found that interferon regulatory factor-8 (IRF8) was the only gene with strong age-associated expression changes in all analyses, decreasing its expression in each cell type.

Materials and Methods

Additional information about study design, materials and methods section are provided in Supplement 1.

Acquisition of samples from healthy human donors

Bone marrow (BM), mobilized peripheral blood stem cells (PBSC), and peripheral blood (PB) were obtained under Institutional Review Board approved protocols

according to the Declaration of Helsinki. Details about the donor information are provided in Supplement 2. CD34+ cells were selected from BM (BMCD34+) and PBSC (PBCD34+) using anti-CD34 immunomagnetic beads (Miltenyi Biotec, Auburn, CA), yielding highly enriched CD34+ products.(7) PBCD34+ samples with adequate amounts of material were further purified into a highly enriched population of PBCD34+/38- cells by flow-cytometiric sorting. Naïve T-cells were obtained through negative selection and expanded to obtain enough cells for RNA extraction.(8)

RNA extraction, hybridization, and microarrays analyses

RNA from human samples was extracted and was *not* pooled for studies.(9, 10) For BMCD34+ and T-cells, biotin-labeling of total RNA (5 µg) was performed as per standard Affymetrix protocol.(10) For the PBCD34+/38- arrays, the single-stranded linear amplification protocol was used to process RNA (100 nanograms) for the microarrays.(10) Biotin-labeled fragmented antisense cRNA (15 µg) was hybridized to HG-U133A arrays (Affymetrix, Santa Clara, CA).(10) Expression profiles of murine HSCs were available from two data sets: Rossi et al (GEO, GEO accession: GSE 4332) and Chambers et al. (GEO, GEO accession: GSE 6503).(5, 6)

Log_2 expression signals were generated using robust multi-array average (gcRMA) and analyzed in GenePlus™ software (Enodar Biologic, Seattle, WA).(9, 11) All arrays met previously published quality control guidelines.(9) Demographic data and CEL files for the human data are available at the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo). Age-associated expression changes were deemed to be statistically significant if their Z-scores were ≥ 4.75 or ≤ -4.75.(12, 13) Statistically

significant genes with age-associated expression changes were loaded into Ingenuity® Pathways Analysis (IPA) software to determine the involvement of functional pathways (Ingenuity® Systems, www.ingenuity.com).(9)

Quantitative RT/PCR and western blots for IRF8 expression

Quantitative reverse transcription/polymerase chain reactions (Q-RT/PCR) were performed using universal TaqMan® conditions (Applied Biosystems, Foster City, CA).(9, 14) Copy numbers were computed using standard curves, corrected for RNA quality using beta-2 microglobulin (B2M) copy number, and normalized to the median T-cell expression.(15) Two-tailed Student's t-tests and linear regression analyses were performed to determine statistical significance. Western blots were performed using 20 µg of protein.(15) Semi-quantification of the IRF8 protein was performed using ImageJ software.(16)

Results and Discussion

Age-associated expression changes in human CD34+ and CD34+38- cells

Microarray analyses of the BMCD34+ samples (N = 8, age range 16 – 51) and PBCD34+/38- samples (N = 4, age range 22 – 53) identified 161 and 917 genes, respectively, with age-associated expression changes (Supplement 3). The majority of these genes (approximately 65%) displayed an increased expression with age in both populations. Ten genes had similar age-associated expression changes in both data sets (Figure 1a and Supplement 3). Functional pathways affected most significantly by

aging included gene expression, cellular proliferation, cancer, and cellular development (Supplement 3).

Overlap of age-associated expression changes between human CD34+ cells and murine HSCs

Although the human BMCD34+ and PBCD34+38- cells were enriched for HSCs, these CD34+ cells do not represent a pure population of LTR HSCs. Recently, Rossi et al. and Chambers et al. isolated near-homogeneous populations of murine LTR HSCs and examined their age-associated expression changes.(5, 6) Although these murine studies have provided important insights into the age-associated expression changes in HSCs, interspecies differences have been reported.(17) We wondered which age-associated expression changes in the human CD34+ cells also occurred in murine LTR HSCs, believing that genes with age-associated expression changes conserved across species would be of particular interest for future functional studies.

Therefore, we examined the expression profiles of murine HSCs from the Rossi and Chambers studies.(5, 6) Analyses for each data set were performed independently of one another, since different murine array platforms were used. We identified 494 and 3359 genes with age-associated expression changes from the Rossi and Chambers studies, respectively. Eighty-four genes displayed age-associated expression changes in both murine data sets (Supplement 4). We then determined those genes with age-associated expression changes in human CD34+ cells that had at least one probe set represented on either the murine 430-2 and/or MOE430 arrays. Approximately 91% (147 of 161) and 86% (789 of 917) of the genes with age-

associated expression changes in human BMCD34+ and PBSC34+38- cells, respectively, had probe sets on at least one of murine arrays (Supplement 3). Examining the overlap of genes with significant expression changes with aging conserved across human CD34+ cells and murine HSCs, only one gene, IRF8, displayed an age-associated expression change in all four analyses (Figure 1), decreasing its expression with aging in all cell types examined (Supplement 4).

Age-associated expression changes in human T-Cells

To determine if similar age-associated expression changes occurred in more differentiated hematopoietic cells, we compared the expression profiles of expanded T-cells from younger (age ≤ 30) and older (age > 70) normal donors. The analyses identified 13 genes with age-associated expression changes (increased = 4 and decreased = 9, Supplement 5). IRF8 was one of the 13 genes with age-associated expression changes in human T-cells. As with the human CD34+ cells and murine LTR HSCs, IRF8 expression was significantly decreased in older adults.

Validation of decreased IRF8 expression in aging human hematopoietic cells

Q-RT/PCR studies using Taqman assays found that IRF8 expression decreased with aging in BMCD34+ (N = 6, R = 0.77, p = 0.07), PBCD34+38-, (N = 2, p = N/A), and T-cells (N = 26, p = 0.001) (Supplement 6). Thus, the Q-RT/PCR studies confirm the microarray data and age-associated decrease in IRF8 mRNA expression in human hematopoietic progenitor/HSCs and T-cells. Studies in cell lines have found that IRF8 mRNA and protein expression parallel one another, such that IRF8 mRNA expression

may provide a reasonable estimate of relative amount protein expression.(18) However, to assess the relationship between mRNA and protein expression in non-immortalized human hematopoietic cells, we simultaneously examined IRF8 mRNA and protein expression in the PBCD34+ samples from 5 donors. Q-RT/PCR and protein assays (Figure 2a) demonstrated a simultaneous age-associated decrease in IRF8 expression (mRNA, Figure 2B, $R = 0.90$, $p = 0.04$ and protein, Figure 2b, $R = 0.91$, $p = 0.03$). Together, the data indicates IRF8 mRNA and protein expression parallel one another in non-immortalized cells, and its protein expression significantly decreases in human CD34+ cells with aging.

Conclusion

To our knowledge, this is the first report to identify IRF8 as a potential biomarker of aging. Likewise, it is the first study to compare the age-associated expression changes between human CD34+ cells and murine HSCs. Previous studies have established that IRF8, a transcription factor, plays a critical role in normal hematopoiesis, controlling the transcription of several pivotal regulatory genes (NF1, BCL2, $p15^{Ink4b}$).(19-21) Inactivation of IRF8 causes a chronic myeloid leukemia-like syndrome in mice, (22-24) and leukemic blasts frequently display decreased IRF8 expression, suggesting a possible link to malignant transformation in humans.(25, 26) It is intriguing to postulate that perhaps the loss of IRF8 expression in the aging hematopoietic cells *may* contribute to the skewing toward myeloid commitment of HSC and/or increased development of hematopoietic diseases in older adults. However, additional studies are needed to better characterize the age-associated expression

changes of IRF8 in other hematopoietic cells (e.g. monocytes, B-cells, etc.) and to examine the functional consequences of its decreased expression with aging.

Figure Legends

Figure 1. Overlap of Age-Associated Expression Changes in Human CD34+ cells and Murine HSCs. a. Shows the overlap of significant genes with age-associated expression changes between human CD34+ cells from the BM (BMCD34+) and human CD34+38- cells from mobilized stem cell products (PBCD34+38-). Ten genes displayed similar age-associated expression changes in both analyses. **b.** Shows the overlap of significant genes with age-associated expression changes between human BMCD34+ and murine HSCs from the Chambers and Rossi data sets. Two genes (IRF8 and RAB40B) displayed similar age-associated expression changes in all three analyses. **c.** Shows the overlap of significant genes with age-associated expression changes between human PBCD34+38- and murine HSCs from the Chambers and Rossi data sets. Three genes (IRF8, NDRG1, and NEO1) displayed similar age-associated expression changes in all three analyses.

Figure 2. Age-Associated Expression Changes of IRF8 Protein and mRNA Levels in PBCD34+ Cells. a. Western blot for IRF8 (upper blot) and GAPDH (lower blot) was performed using protein lysates (20 µg) from 5 PBCD34+ samples. IRF8 expression appeared to be highest in the 27 year old donor and lowest in the 63 and 72 year old donors. **b.** Q-RT/PCR was performed in triplicate using RNA from the same 5 samples. IRF8 mRNA expression (y-axis, right side) corrected for B2M and adjusted to median T-cell expression is show for each sample (closed circles, •). Regression analysis revealed a significant decrease in expression with donor age ($R = 0.90$, $p = 0.04$). Semi-

quantification of IRF8 protein expression was performed using the western blot from Figure 2a and ImageJ software. IRF8 protein expression was corrected for GAPDH signal. The relative amount of corrected IRF8 protein signal is shown for each sample (closed squares, ■). As with the mRNA data, regression analysis revealed a significant age-associated decrease in IRF8 protein expression ($R = 0.91$, $p = 0.03$).

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