The biology of human hematopoietic stem and progenitor cells in acute myeloid leukemia, aging and autologous transplantation

Woolthuis, Carolien Marthe

CHAPTER 6

Aging impairs the long-term hematopoietic regeneration after autologous stem cell transplantation

Carolien M. Woolthuis[1], Niccolo Mariani[1], Rikst Nynke Verkaik-Schakel[1], Annet Z. Brouwers-Vos[1], Jan Jacob Schuringa[1], Edo Vellenga[1], Joost Th. M. de Wolf[1*], Gerwin Huls[1*]

[1] Department of Hematology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

* these authors contributed equally to this work.

In progress

Abstract

Aging of the hematopoietic system is especially studied in animal models. To reveal the potential effects of ontogeny and aging on human hematopoietic stem and progenitor cells (HSPC) $CD34^+$ cells derived from cord blood, young adult bone marrow and old adult bone marrow were investigated with respect to phenotype, *in vitro* function and gene expression profiles. We observed an increased frequency of phenotypically defined HSPCs upon aging, but no distinct differences in *in vitro* function or gene expression. Since regeneration of peripheral blood counts can be considered a read-out of HSPC function, we compared various peripheral blood cell parameters between younger (≤50 yrs, n=64) and older patients (≥60 yrs, n=55) after autologous stem cell transplantation (ASCT). The age of the patients did not affect the number of apheresis cycles nor the amount of $CD34^+$ cells that were harvested. The parameters for short-term regeneration (granulocytes $>0.5x10^9$/L, thrombocytes $>20x10^9$/L) did not differ significantly between the younger and older patients. However, complete recovery of all three peripheral blood lineages one year after transplantation was strongly affected by advanced age and occurred in 56% of younger and only 29% of older patients (p=0.009). These data suggest that aging has only limited effects on $CD34^+$ human HSPCs in steady state conditions, but can become important in situations of chemotoxic and replicative stress.

Introduction

The bone marrow is one of the most highly self-renewing tissues of the body. Hematopoietic stem cells (HSCs) are capable of replenishing all cell types of the blood during the complete lifespan of an organism. Nevertheless, like other tissues, the hematopoietic system does not escape the effects of aging. In humans, the effects of aging on the hematopoietic system are clinically manifested by an increase in the incidence of myeloproliferative diseases, including leukemia,[1;2] a decline in adaptive immunity,[3-5] and a greater propensity to anemia[6;7]. Experimental studies in the murine system comparing young versus old HSCs demonstrate a skewing towards a more myeloid-biased output,[8-10] a relative increase in phenotypical defined HSCs[9-13] and a decrease in competitive repopulating ability of old versus young murine HSCs[9-11].

Until recently, only limited data was available on age-associated changes in human HSCs. Two recent studies have addressed this issue and suggest that, although some of the findings observed in the murine system could be confirmed in humans, differences between young and old human HSCs might not be as distinct. Phenotypical analyses by Kuranda et al. demonstrate a significant increase in the number of multipotent $CD34^{+}CD38^{-}$ cells with age together with decreases in the frequency of B/NK precursors and committed B-lymphoid progenitors.[14] However, functional experiments did not reveal significant differences in the level of human chimerism or the frequency of NSG-mice repopulating cells between $CD34^{+}$ bone marrow cells isolated from young and elderly individuals.[14] A study by Pang et al. characterizing the age-associated effects on human hematopoietic stem and progenitor cells (HSPCs), also reported an increase in the frequency of phenotypically defined HSCs with age. After transplantation to NSG mice a slight impairment in engraftment efficiency of elderly HSCs and a myeloid bias of HSCs with aging was observed.[15]

A better understanding of HSC aging will facilitate the development of treatment regimens that are more suitable for elderly patients undergoing stem cell transplantation and other stem cell mediated therapies. In this study, $CD34^{+}$ cells derived from cord blood, young adult bone marrow and old adult bone marrow were investigated with respect to phenotype, *in vitro* function and gene expression profiles. We considered autologous HSPC transplantation as an experimental opportunity to study the impact of aging and stress response on human HSPCs *in vivo*. Therefore, we have analysed the impact of age on mobilization of HSPCs and the impact of age on the short and long-term regeneration after autologous stem cell transplantation (ASCT).

Material and methods

Normal bone marrow and cord blood material

After achieving informed consent bone marrow aspirate was obtained from: (a) Patients older than 60 years of age who receive a total hip replacement, (b) Volunteers younger than 35 years of age (and older than 18 years), (c) Healthy potential donors for hematopoietic stem cell transplantation who undergo a bone marrow aspirate as part of the standard medical examination. The protocol for bone marrow collection was approved by the institutional review board (IRB) of the University Medical Center Groningen (UMCG). All participants had normal general health, normal peripheral blood counts and did not suffer from a haematological disorder. Neonatal cord blood (CB) was obtained after informed consent from healthy full-term pregnancies from the obstetrics departments of the UMCG and the Martini Hospital Groningen, the Netherlands.

Flow cytometry analysis and sorting procedures

The mononuclear cell (MNC) fraction from CB and BM was isolated by density gradient centrifugation using lymphoprep (PAA, Cölbe, Germany). $CD34^+$ cells were enriched by MiniMACS (Miltenyi Biotec, Amsterdam, the Netherlands) selection according to manufacturer's instructions. Sorting of $CD34^+$ cells for the *in vitro* assays was performed by MoFLo sorting (Dako Cytomation, Carpinteria, CA, USA) using a CD34 PE-labeled antibody (Clone 8G12, BD Biosciences, San Jose, California, USA). The fluorescence activated cell sorting (FACS) analyses were performed on an LSR II flow cytometer (Becton Dickinson (BD), Alpen a/d Rijn, The Netherlands). Antibodies were obtained from BD. Data were analyzed using FlowJo (Tri Star, Inc, Ashland, OR, USA) software.

Gene expression profiling

Total RNA was isolated using the RNeasy micro kit from Qiagen (Venlo, The Netherlands) according to the manufacturer's recommendations. RNA quality was examined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Genome-wide expression analysis was performed on Illumina (Illumina, Inc., San Diego, CA) BeadChip Arrays Sentrix Human-12 v3 (46k probesets). Typically, 200ng mRNA for amplification with Illumina TotalPrep RNA Amplification Kit (Ambion) and 750ng of cRNA was used in labeling reactions and hybridization with the arrays according to the manufacturer's instructions. Data were analyzed using the BeadStudio v3 Gene Expression Module (Illumina, Inc.) and Genespring (Agilent, Amstelveen, The Netherlands).

In vitro culture assays

The colony forming cell (CFC) assay was performed in methylcellulose (MethoCult H4230, StemCell Technologies, Grenoble, France), supplemented with 20 ng/ml IL-3, 20 ng/ml IL-6, 20 ng/ml G-CSF, 20 ng/ml c-Kit ligand and 1 U/ml EPO (Cilag Eprex, Brussels, Belgium) as previously described.[16]

The assay was performed in duplicate. Two weeks after plating CFU-GM (colony forming unit - granulocyte/macrophage), BFU-E (burst forming unit - erythroid) and CFU-GEMM (colony forming unit - granulocyte erythrocyte monocyte megakaryocyte) were scored. For the long-term cultures $CD34^+$ cells were plated in bulk in 12-wells plates or limiting dilution in a 96-wells plate precoated with MS5 stromal cells. Cells were expanded in LTC medium (α-minimum essential medium supplemented with heat-inactivated 12.5% fetal calf serum (Sigma, Zwijndrecht, The Netherlands), heat-inactivated 12.5% horse serum (Sigma), penicillin and streptomycin, 2 mM glutamine, 57.2 μM β-mercaptoethanol (Sigma) and 1 μM hydrocortisone (Sigma)) supplemented with 20 ng/ml IL-3, 20 ng/ml granulocyte colony-stimulating factor (G-CSF) (Rhone-Poulenc Rorer, Amstelveen, The Netherlands) and 20 ng/ml thrombopoietin (TPO) (Kirin, Tokyo, Japan). Cultures were kept at 37 ºC and 5% CO_2. Cultures were demi-depopulated weekly for medium change. For the MS5 co-cultures cells were counted weekly. For the LTC-IC limiting dilution assay five weeks of expansion was followed by removing suspension cells and adding methylcellulose (StemCell Technologies) as described for the CFC assay. Two weeks later, wells containing CFCs were scored as positive and the LTC-IC frequency was calculated using L-Calc Limiting Dilution Software (StemCell Technologies). Liquid cultures were performed exactly as described for the co-cultures but without co-culturing MS5.

Transplantation procedure

Peripheral blood stem cells (PBSC) were collected following a disease-orientated course of chemotherapy and granulocyte-colony stimulating growth factor (G-CSF). Harvesting was performed via a dual-lumen catheter using a COBE Spectra apheresis machine. $CD34^+$ cells were stained using a monoclonal anti-CD34 (anti HPCA-2) and quantified by flow cytometry (FACS Calibur, BD). A target quantity of $CD34^+$ cells of more than 2×10^6/kg body weight was envisaged. Routinely, CFU-GM assays were performed of all apheresis products. Therefore 5 x 10^4 total nucleated cells (TNC) were seeded and cultured in small Petri dishes (35 mm diameter) in a volume of 1 ml standard methylcellulose complete medium containing 1% methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml recombinant human (rh) Stem Cell Factor, 20 ng/ml rh GM-CSF, 20 ng/ml rh IL-3, 20 ng/ml rh IL-6, 20 ng/ml rh G-CSF (Methocult, StemCell Technologies). After 14 days incubation at 37ºC in humidified air with 5% CO_2, colonies composed of more than 50 non-erythroid cells were scored as CFU-GM.

None of the studied patients received blood or platelet transfusions in the four weeks preceding ASCT. All patients were treated with selective gut decontamination during neutropenia. Neither G-CSF nor erythropoietin was administered after ASCT. Platelets were transfused if the platelet count fell below 10×10^9/L or if bleeding occurred. Erythrocytes were transfused if the hemoglobin level dropped below 5 mmol/L or if the patient had symptomatic anemia. All blood products were irradiated.

The leukapheresis product was mixed with minimal essential medium (MEM) resulting in a final dimethyl sulphoxide concentration of 10%. The final cell suspension was transferred into freezing bags and frozen to -160 °C with a computer-controlled cryopreservation device. The frozen cells were then transferred into liquid nitrogen and stored at -196 °C. Immediately before infusion, the cryopreserved peripheral stem cells were thawed in a 40 °C water bath and reinfused through a double-lumen Hickman catheter. Premedication consisted of steroids and antihistamines.

Statistical analysis

The Mann-Whitney U or Chi-squared test was used for analysis of individual group differences. Hematological recovery was assessed using Kaplan-Meier probability curves and statistical comparison of curves was performed by the log-rank test. The prognostic value of different variables for probability of hematological recovery was assessed by univariate and multivariate analysis using the Cox multiple-regression model. Differences with a P-value ≤0.05 were considered statistically significant, except for the gene expression profiling where expression differences with a p-value < 0.00001 (Student's t-test) were considered statistically significant.

Results

Phenotypical changes within the hematopoietic system in ontogeny and aging

The hematopoietic system is hierarchically organized with the multipotent stem cells on top, followed along the line by progenitors with differentiation potential along distinct lineages within the myeloid or lymphoid branches. To investigate whether the processes of ontogeny and aging have an effect on the composition of the different stem and progenitor populations within the total population, we performed phenotypical analyses of the total mononuclear cell fraction (MNC) of CB, young adult and old adult BM. No significant differences in the percentages of the total $CD34^+$, the progenitor-containing $CD34^+CD38^+$, the multipotent $CD34^+CD38^-$ fractions were observed between CB and young BM, however the percentages of all these populations increased significantly from young to old BM and between CB and old BM (Figure 1A). In contrast, the percentage of lymphoid progenitors, defined as $CD34^+CD10^+$ within the total $CD34^+$ population, was relatively low in CB, high in young adult BM, but significantly decreased upon aging in adult BM (p=0.021, Figure 1B). Subsequently, the frequency of CMP (common myeloid progenitors), GMP (granulocyte macrophage progenitors) and MEP (megakaryocyte erythrocyte progenitors) within the $CD34^+CD38^+$ fraction was analysed as function of age (Figure 1C). A significantly higher percentage of phenotypically defined GMP cells was observed in old BM compared to CB, but no other significantly differences in the percentage of CMP, GMP and MEP were seen upon aging or ontogeny (Figure 1C).

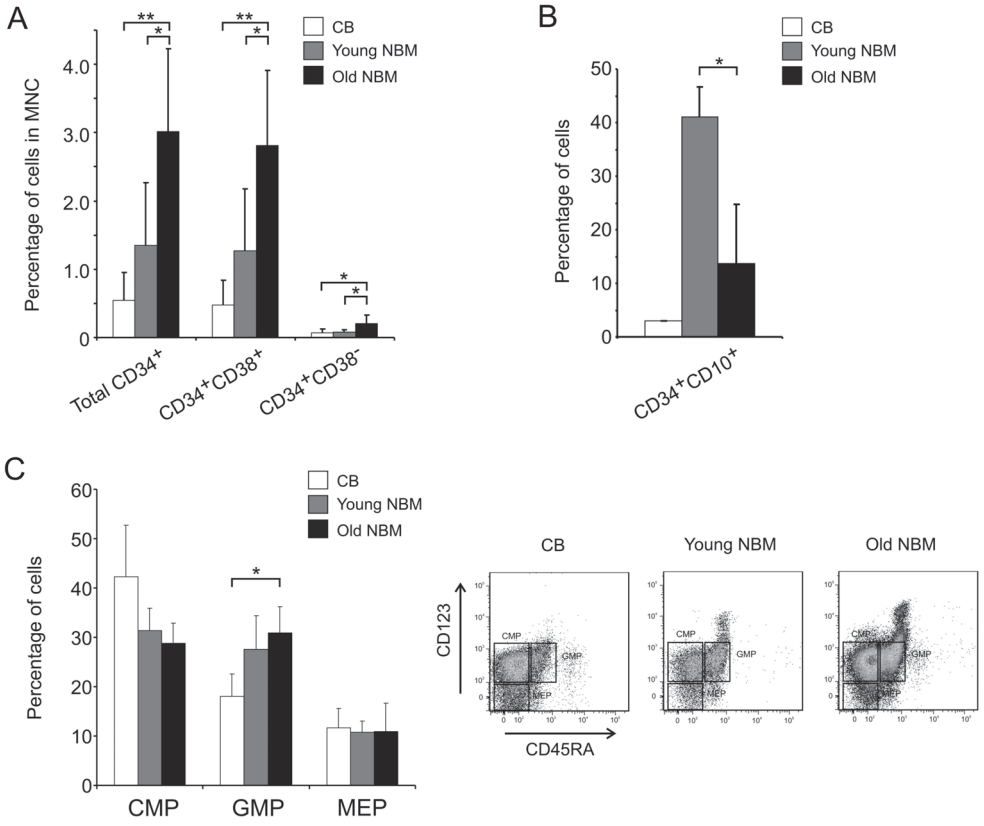


Figure 1 Phenotypical changes upon aging and ontogeny
(A) Percentages of total CD34$^+$, progenitor-containing CD34$^+$CD38$^+$ and multipotent CD34$^+$CD38$^-$ in the mononuclear cell fraction of cord blood (CB, n=5), young normal bone marrow (NBM, n=5) and old NBM (n=8). (B) Percentages of cells with a lymphoid progenitor phenotype defined as CD10$^+$ within the CD34$^+$ population. (C) Percentages of cells phenotypically defined as common myeloid progenitor (CMP), granulocyte macrophage progenitor (GMP) and megakaryocyte erythroid progenitor (MEP) within the CD34$^+$CD38$^+$CD10$^-$ population. On the right representative FACS plots with gating strategy for each sample group. Means ± SD are plotted. * p<0.05, ** p<0.01.

Comparison of gene expression profiles of CD34$^+$ cells derived from cord blood and young and old bone marrow

We next compared CD34$^+$ stem and progenitor cells derived from CB (n=15), young adult bone marrow (n=10) and old adult bone marrow (n=22) with respect to their gene expression by genome-wide microarray analyses. Comparing the transcriptomes of CD34$^+$ young versus old bone marrow cells revealed a limited number of differentially expressed probes (considering a p-value < 0.00001 with Student's t-test), namely 18 upregulated probes, representing 14 annotated genes (Figure 2A).
Compared to CD34$^+$ young adult BM cells, 134 probes were found to be upregulated and 272 probes to be downregulated in CD34$^+$ CB (p-value < 0.00001 with Student's t-test). When CD34$^+$ CB was

compared with old adult BM cells, 555 probes were found to be upregulated and 650 probes to be downregulated. The overlap is pictured in Figure 2B. The results of the gene ontology (GO) analyses for the genes differentially expressed between CD34+ CB and CD34+ adult BM (both young and old) are provided in Figure 2C.

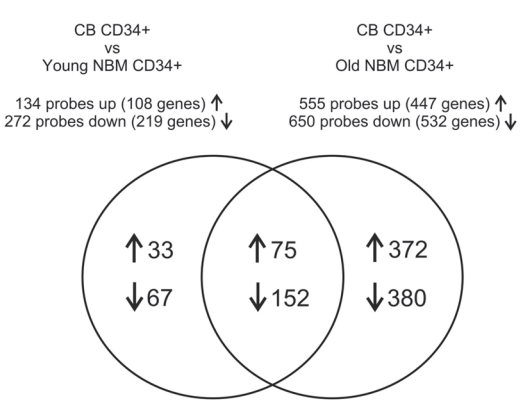
Collectively, the results of the gene expression analyses indicated that transcriptome differences are much more pronounced when comparing CB versus adult bone marrow than between young and old adult bone marrow.

A

Old NBM CD34+
vs
Young NBM CD34+

Probe ID	Gene symbol	fold NBMold/ NBMyoung	ttest NBMold/ NBMyoung
1410113	ARL4A	3.49	3.17E-06
1820594	HBEGF	4.66	3.91E-08
4210315	NA	1.54	9.57E-06
7380100	MCL1	2.95	4.65E-06
2490719	STK17B	4.55	3.71E-06
4490520	GPR183	3.34	9.97E-06
2640735	BHLHE40	2.79	8.08E-06
6760546	TIPARP	2.11	5.04E-06
2680543	PDE4B	2.72	3.44E-06
6400364	NR4A2	5.07	7.10E-07
650639	NR4A2	4.01	5.83E-06
7560615	ARL4A	2.43	7.63E-06
3780370	C9orf72	2.48	5.40E-06
1690678	SIK1	3.64	4.88E-07
5050162	CD83	2.77	9.52E-06
3060682	BPGM	3.87	1.65E-06
1010435	C1orf55	1.85	1.85E-06
1070519	NA	1.62	1.17E-06

B



C

GO terms associated with upregulated genes

Category	Term	Count	PValue	FDR
SP_PIR_KEYWORDS	phosphoprotein	224	1.68E-07	2.33E-04
GOTERM_BP_FAT	GO:0006412~translation	25	2.32E-06	0.003952
SP_PIR_KEYWORDS	chromosomal rearrangement	19	1.09E-04	0.15025
SP_PIR_KEYWORDS	protein biosynthesis	15	1.44E-04	0.198784
GOTERM_MF_FAT	GO:0005083~small GTPase regulator activity	19	2.26E-04	0.328807
UP_SEQ_FEATURE	mutagenesis site	73	3.21E-04	0.52624
GOTERM_MF_FAT	GO:0030695~GTPase regulator activity	23	6.61E-04	0.960114

GO terms associated with downregulated genes

Category	Term	Count	PValue	FDR
GOTERM_BP_FAT	GO:0000279~M phase	72	8.53E-42	1.49E-38
SP_PIR_KEYWORDS	mitosis	54	1.97E-41	2.81E-38
SP_PIR_KEYWORDS	cell division	62	3.04E-41	4.34E-38
SP_PIR_KEYWORDS	cell cycle	78	3.05E-41	4.36E-38
GOTERM_CC_FAT	GO:0005819~spindle	36	1.89E-22	2.62E-19
GOTERM_CC_FAT	GO:0000793~condensed chromosome	32	3.90E-20	5.42E-17
SP_PIR_KEYWORDS	kinetochore	21	3.18E-17	4.54E-14
GOTERM_CC_FAT	GO:0000775~chromosome, centromeric region	28	1.60E-16	1.55E-13
GOTERM_BP_FAT	GO:0007017~microtubule-based process	34	5.64E-13	9.85E-10
GOTERM_BP_FAT	GO:0048015~phosphoinositide-mediated signaling	15	2.57E-07	4.49E-04
GOTERM_BP_FAT	GO:0007010~cytoskeleton organization	33	1.90E-06	0.003314

Figure 2 Gene expression profiling

(A) Probes differentially expressed between CD34+ cells derived from old and young adult normal bone marrow (NBM). NA=not annotated. (B) VENN diagram for the overlap in differentially expressed probes between CD34+ cells derived from CB and either young NBM (left) or old NBM (right). The numbers of annotated genes represented by the probes are written between parentheses. (C) Results of the gene ontology analyses for the differentially expressed genes between CD34+ CB and CD34+ NBM (both young and old). FDR= false discovery rate.

Impact of ontogeny and aging on function of hematopoietic stem and progenitor cells in vitro

To study the functional capacity of $CD34^+$ cells derived from CB and young and old adult BM, several *in vitro* assays were performed. The *in vitro* colony forming cell assay (CFC) revealed a higher colony forming potential for CB compared to both young and old bone marrow, which was characterized by a higher output of CFU-GM and CFU-GEMM colony formation (Figure 3A). No significant differences between the groups were found for the BFU-E colony formation. Colony forming potential of $CD34^+$ cells for all included lineages was very much comparable between young and old bone marrow. Also the LTC-IC frequency, an established *in vitro* measurement for hematopoietic stem cells, was comparable between young and old adult BM derived $CD34^+$ cells (Figure 3B). To further assess the impact of age on self-renewal and differentiation potential of $CD34^+$ human cells, these cells were cultured in liquid and MS5 co-cultures. As indicated in Figure 3C CB derived $CD34^+$ cells had a proliferative advantage compared to adult BM in both assays. As for the other *in vitro* assays, no significant difference in growth was observed between $CD34^+$ cells derived from young and old adult BM. In addition, we did not observe differences in serial replating capacity assessed in the MS5 co-cultures between young and old BM, while the serial replating capacity of CB was increased compared to adult BM (data not shown).

Taken together, these data indicate that CB derived $CD34^+$ cells have a proliferative advantage compared to adult BM, whereas no differences in proliferation could be observed between young and old $CD34^+$ BM cells as examined by the different *in vitro* assays.

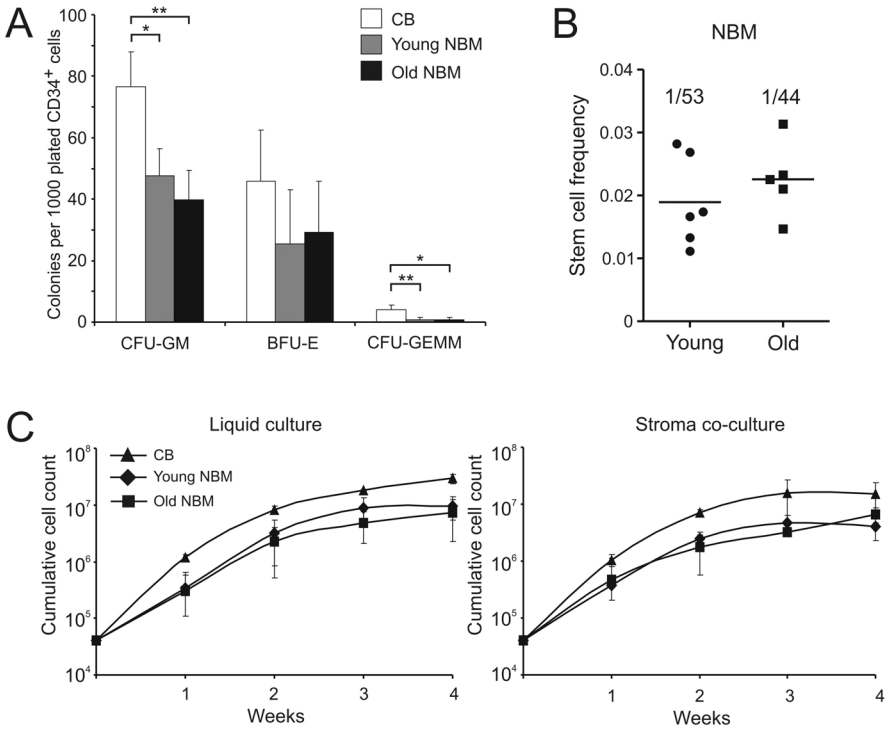


Figure 3 In vitro function of hematopoietic stem and progenitor cells
(A) Results of *in vitro* colony forming cell assay (CFC) for CB (n=5) compared to both young (n=6) and old bone marrow (n=5). Bars represent mean number of colonies ± standard deviation (SD). (B) LTC-IC frequencies of one representative sample per group. Lines indicate trend lines for the whole group and written frequencies are the mean frequencies per group of young BM (n=6) and old BM (n=5). (C) Results of liquid culture (left) and MS5 co-cultures (right) for CB (n=2), young BM (n=4) and old BM (n=4). Means ± SD are plotted. * $p<0.05$, ** $p<0.01$.

Hematopoietic stem and progenitor cells in vivo

To follow-up on our *in vitro* research on the impact of aging on human hematopoietic stem and progenitor cell function, we extended our research to an *in vivo* situation, namely that of an autologous stem cell transplantation (ASCT). In this setting we studied mobilization and apheresis of peripheral blood stem cells and compared regeneration data in younger and elderly patients after ASCT.

Table 1 Patient characteristics per age group

	≤ 50 yrs	≥ 60 yrs	P-value
Total (n)	64	55	
Non Hodgkin Lymphoma (n)	27	18	
Multiple Myeloma (n)	37	37	
Age (mean)	44 ± 7	63 ± 2	< 0.0001
Male (%)	65	57	0.5
Non Hodgkin lymphoma			
Number of chemotherapy courses before mobilization	7.2 ± 3.1	6.6 ± 2.9	0.5
(R)-CHOP (%)	100	100	1.0
Mobilizing chemotherapy			
(R)-DHAP (%)	77	50	0.2
High Dose Ara-C (%)	12	38	0.06
CHOP (%)	12	0	0.5
Cyclophosphamide (%)	0	6	0.5
G-CSF alone (%)	0	6	0.5
Conditioning chemotherapy			
BEAM (%)	100	100	
Multiple myeloma			
Number of chemotherapy courses before mobilization	3.3 ± 0.8	4 ± 3.4	0.2
VAD (%)	56	67	0.5
PAD (%)	25	16	0.6
TAD (%)	19	17	1.0
Mobilizing chemotherapy			
CAD (%)	62	75	0.3
Cyclophosphamide (%)	38	25	
Conditioning chemotherapy			
High dose Melphalan (%)	95	94	1.0
Cyclo/TBI (%)		6	
BEAM (%)	5		

Patient characteristics

In total 119 patients were studied. All subsequent multiple myeloma (MM) and non-Hodgkin (NHL) lymphoma patients younger than 50 years or older than 60 years treated with high dose chemotherapy (HDC) followed by ASCT at the department of Hematology, UMCG, between June

1993 and June 2007 were included for analysis, with the exception of patients who had progressive disease or relapsing disease within one year after transplantation. Within the NHL group only patients with diffuse large B cell lymphoma (DLBCL) and mantle cell lymphoma (MCL) were included. The younger age group (≤ 50 years) consisted of 64 patients (mean age 44 ± 7 years) of which 27 were diagnosed with NHL and 37 with MM. The older age group (≥ 60 years) included 55 patients (mean age 63 ± 2 years) and consisted of 18 NHL patients and 37 MM patients. All MM patients received their ASCT upfront after induction therapy with VAD (Vincristine, Adriamycin, Dexamethason) or TAD (Thalidomide, Adriamycin, Dexamethason).[17-19] All NHL patients received their ASCT at second line, after first line treatment with 6-8 cycles of (R)-CHOP chemotherapy. Re-induction treatment consisted of (R)DHAP-(R)VIM-(R)DHAP chemotherapy. Patients with MM were conditioned with high-dose melphalan (200 mg/m^2) (n=70) cyclophosphamide + TBI (n=2) or BEAM (n=2). Patients with NHL were conditioned with BEAM (n=45). Patient characteristics are listed in Table 1.

Table 2 Results of apheresis and regeneration of peripheral blood counts after ASCT

	≤ 50 yrs	≥ 60 yrs	P-value
Apheresis			
CD34$^+$ cells collected at day 1 (x10^6/kg)			
Multiple myeloma (median)	11	13	0.3
Non-Hodgkin lymphoma (median)	11.6	9.6	0.7
Patients reaching targeted CD34$^+$ cell number by mobilization at day 1 (%)	59	64	0.6
at day 2 (%)	30	23	0.5
at day 3 (%)	8	7	0.9
at day 4 (%)	3	4	0.9
Transplantation			
Mean number of infused CD34$^+$ cells (x10^6/kg)	8.9 ± 9.7 (median 5.7)	6.2 ± 4.8 (median 5.0)	0.1
Regeneration			
Granulocytes ≥ 0.5 x10^9/L (days)	18.5 ± 11.1 (median 16)	22.8 ± 20.9 (median 16)	0.2
Patients with granulocytes ≥ 0.5 x10^9/L at day 28 (%)	4.9	18	0.03
Thrombocytes > 20 x10^9/L	28.2 ± 46.8 (median 17)	29.4 ± 25.5 (median 19)	0.9
Patients with thrombocytes > 20 x10^9/L at day 40 (%)	11.5	20.4	0.3
Patients with normal peripheral blood counts of all three lineages (Hb, leukocytes and thrombocytes) after 1 year (%)	56	29.6	0.009

Mobilization and apheresis

Different mobilizing therapy regimens were used, but percentages of patients receiving one of these therapies did not differ significantly between age groups (Table 1). For patients with NHL the aim was to harvest 5 x 10^6 $CD34^+$ cells/kg and for MM patients the aim was to harvest 10 x 10^6 $CD34^+$ cells/kg. The number of apheresis days to reach these targets was not significantly different in the younger compared to the older age group (Table 2). Moreover, no significant differences in median number of $CD34^+$ cells collected at the first day of apheresis could be observed between younger and older patients. At the first day of apheresis 11 x 10^6 $CD34^+$ cells/kg and 13 x 10^6 $CD34^+$ cells/kg were collected for younger and older patients with MM respectively. For patients with NHL the younger patients collected 11.6 x 10^6 $CD34^+$ cells/kg and the older patients 9.6 x 10^6 $CD34^+$ cells/kg (Table 2).

No age-related effect on the colony forming potential of PBSC material

Routinely, CFU-GM assays were performed on a small aliquot of the collected autologous transplant. Of all 119 patients in our analyzed cohort CFU-GM data were available. In the younger group 34 ± 29 (median 28) x 10^4/kg CFU-GM were administered versus 33 ± 23 (median 29) x 10^4/kg CFU-GM in the older patients. The number of CFU-GM was not significantly different between MM or NHL patients younger than 50 years compared to older than 60 years. In addition, we could not reveal an inverse correlation between age and CFU-GM colony formation (data not shown).

Regeneration of peripheral blood cell counts after ASCT

In order to get insight in the *in vivo* function of the $CD34^+$ cells used for ASCT, various peripheral blood cell parameters were compared between younger and older patients. All the studied parameters are considered to be representative for the short-term hematological recovery after transplantation. The mean number of days after stem cell infusion needed to reach a granulocyte count of at least 0.5 x 10^9/L was 18.5 (± 11.1) days in the younger age group and 22.8 (± 20.9) days in the older age group (Table 2) and did not differ significantly between both groups. A significant difference was observed in the percentage of patients with a granulocyte count of 0.5 x10^9/L or higher at day 28. This was 18% of the elderly patients compared to 4.9% of the younger patients (p=0.03). As was true for the granulocytes, the mean number of days after stem cell infusion needed to reach a platelet count of at least 20 x 10^9/L, without platelet transfusions, was not significantly different between younger and older patients (Table 2).

While younger and older patients were transplanted with comparable numbers of $CD34^+$ cells, one year after transplantation a significant amount of the older patients did not have a complete recovery of their peripheral blood counts. Of the younger patients 56% of patients had normal peripheral blood counts one year after transplantation compared to only 29% of the older patients (P=0.009 and Figure 4). Interestingly, the effect of age on complete recovery of peripheral blood counts one year after transplantation was most pronounced in patients with NHL. Of the younger NHL patients 40%

had complete recovery of peripheral blood counts compared to 0% of the older patients one year after transplantation (Figure 4).

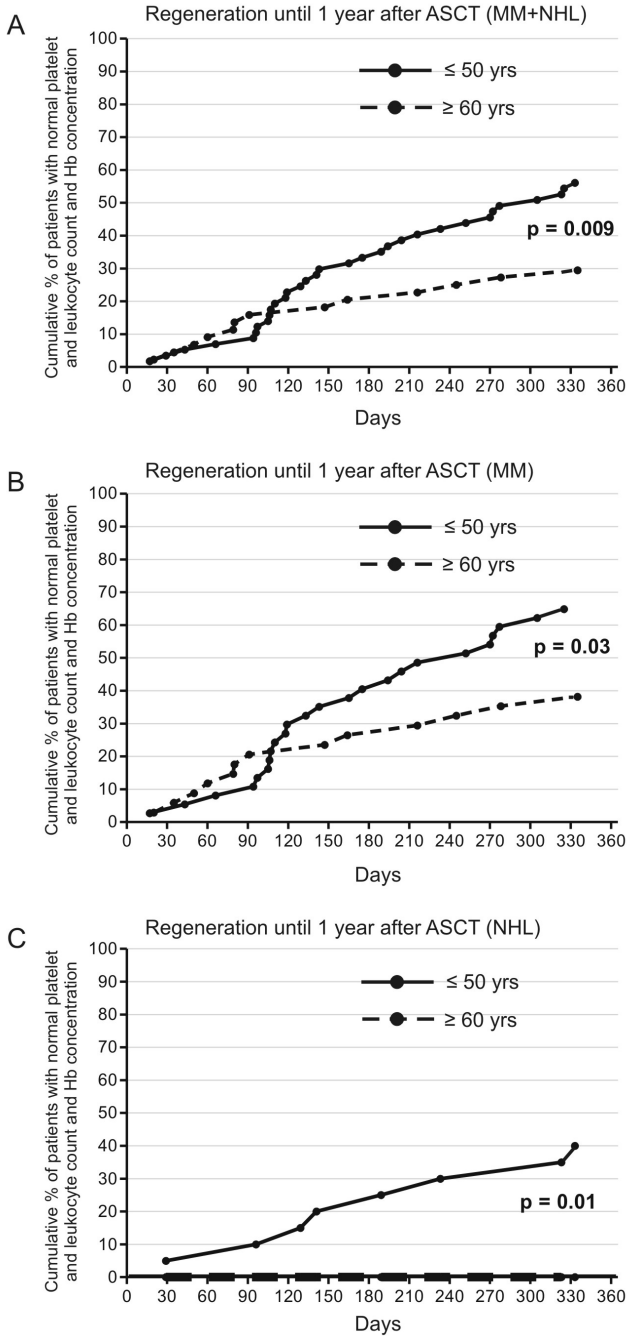


Figure 4 Regeneration of peripheral blood cell counts during one year after ASCT

Graphs present the percentages of patients with normal platelet and leukocyte counts and normal hemoglobin (Hb) level in peripheral blood during one year after ASCT according to age group. In (A) all 119 patients are included, subdivided in (B) for multiple myeloma (MM) patients (n=74) and in (C) for non-Hodgkin lymphoma (NHL) patients (n=45). P-values consider the differences between both age groups at one year of follow-up.

Discussion

Our observations obtained by studying the phenotype, gene expression profile and *in vitro* capacities of both CB as well as adult bone marrow suggest that the process of ontogeny has a greater impact on these characteristics than the process of aging. This is probably most clearly illustrated by the results of the gene expression profiling. Genome-wide microarray analysis comparing young versus old adult BM revealed a very limited number of differentially expressed genes, whereas the differences between CB and adult BM were much more pronounced. Moreover, while we observed an enhanced proliferation and expansion potential of $CD34^+$ cells derived from CB compared to $CD34^+$ cells derived from adult BM, no differences in the *in vitro* capacities could be observed between younger and older adult BM. Importantly, the performed phenotypical analyses did reveal significant differences between young and old adult BM including a strong decrease in phenotypically defined lymphoid progenitors in elderly individuals. These latter results are in line with previously published data in both mice and human.[8-10;14;15]

Considering the situation of ASCT in humans as a model for assessing the effect of increasing age and stress response on HSPC function *in vivo*, we compared clinical mobilization, apheresis and regeneration data between younger and older patients. While no significant differences could be observed between younger and older patients with respect to mobilization and apheresis parameters, a strong impairment in peripheral blood regeneration levels was observed for elderly patients one year after ASCT.

Increasing age of the hematopoietic system is clinically illustrated by an impairment of the adaptive immune system,[3-5] the onset of anemia in elderly,[6;7] and an increased incidence of myeloid diseases including myelodysplasia and leukemia[1;2;20]. Studies in the murine system have suggested that these phenotypic changes in the hematopoietic system might be caused by HSPC intrinsic factors, HSPC extrinsic factors (associated with an aging micro-environment) and/or by changes in the clonal composition of the HSPC compartment.[21] Still, only limited biological data is available concerning the impact of age on the HSPC compartment in humans. In agreement with data reported by others,[14;15;22;23] we observed a relative increase in the percentage of both $CD34^+CD38^-$ and $CD34^+CD38^+$ cells in adult BM upon aging. These human studies do not provide any information on the absolute number of HSCs in younger and older bone marrows, especially since bone marrow cellularity was suggested to decrease upon aging.[24;25] The function of $CD34^+$ cells derived from bone marrow of younger or older individuals was not different in various *in vitro* assays (CFC, liquid culture and MS5 co-culture, LTC-IC) suggesting that the increase in phenotypically defined stem cells is not accompanied by an increase in functional HSCs.

Apparently, $CD34^+$ BM derived cells from older and younger individuals are quite comparable under steady state conditions. It could also be argued that the *in vitro* assays used in the present study are not sensitive enough to detect potential age-related effects, but only limited variation between

samples within one group was observed in the *in vitro* read-outs. Moreover, recent *in vivo* transplantation studies in NOD/SCID/Il-2Rγ null (NSG) mice with young and old human BM by others were not consistent. One study did not reveal differences in the frequency of NSG repopulating cells with increasing age,[14] while another study reported a significant difference (after transplantation of 500 HSC) in the frequency of NSG repopulating cells with age, from approximately 2% to approximately 1%.[15]

Different hypotheses could be proposed to explain the difference observed between murine and human studies with respect to the effect of age on HSCs. One possibility could be searched in limitations on stringent purification of human HSCs compared to murine HSCs. Potential differences could therefore be masked by contaminating progeny. Another hypothesis would be that $CD34^+$ BM derived cells from older and younger individuals are comparable under steady state conditions and that potential differences will only become apparent in stressful conditions, for example in the case of serial transplantation with limited cell numbers or chemotherapy treatment. From this perspective, we have considered ASCT an experimental opportunity to study the impact of age on human HSC function *in vivo* without xenogeneic immunological or metabolic effects. Moreover, the ASCT situation is a clinically relevant setting. Hematopoietic stem cell transplantation is the only well-established clinically applied stem cell based therapy. Elderly patients are the demographic group with the greatest need for this form of treatment. The clinical relevance is also illustrated by a previous study in a cohort of 98 patients with hematological malignancies treated with high-dose chemotherapy followed by ASCT revealing that only 39% of patients had complete recovery of all three lineages after one year.[26] The disadvantage of our approach is that regeneration is not only dependent on age of the transplanted HSPCs, but also on the effects of intensive chemotherapy on both the HSPCs and on the bone marrow micro-environment. Further, as could be true for transplantation studies in NSG mice, age-related differences in homing capacity and micro-environmental changes might impact on regeneration. On the other hand, repetitive cycles of chemotherapy might be a model to induce accelerated aging and the observed age-related effects might be enlarged and therefore better appreciated. We indeed observe a strong impact on long-term regeneration after ASCT, but not on short-term regeneration, suggesting that older HSCs are more hampered than younger HSCs by the procedure of chemotherapy, mobilization, apheresis, transplantation and regeneration. This effect seems more pronounced after more intensive chemotherapy. Patients with NHL received significantly more chemotherapy courses before mobilization of stem cells compared to patients with MM (younger patients $p<0.0001$, older patients $p=0.007$) and showed a more significant effect of age on long-term regeneration.

The upper age limit for ASCT is constantly increasing.[27;28] One of the factors directly affecting the feasibility of ASCT is the mobilization capacity of $CD34^+$ cells. The available data on the effect of increasing age on mobilization are somewhat inconsistent. Several studies demonstrate poorer mobilization in elderly patients, but although the quantity of $CD34^+$ cells collected in older patients

was lower, this quantities did reach the standard target level requested for performing one or more ASCT in most studies.[29-31] Therefore, advanced age is not an obstacle for the collection of a stem cell product capable of restoring hematopoietic function. In our cohort no quantitative differences were observed in number of mobilized and collected $CD34^+$ cells after apheresis. The discrepancy with previous studies might be due to the relative low number of patients studied, the fact that the patients were mobilized after a course of chemotherapy or the volume of peripheral blood that was used for apheresis. Interestingly, studies in mice have shown that mobilization efficiency of primitive hematopoietic cells from aged mice was approximately 5-fold higher, presumably due to reduced adhesion of hematopoietic progenitor cells to bone marrow stroma.[32] The effects of age on homing of human primitive hematopoietic cells remain poorly understood. In murine models a reduced seeding efficiency of old murine HSPCs compared with young HSPCs has been observed.[13;33] Moreover, it has been observed, using time-lapse 2-photon microscopy, that aged early hematopoietic progenitor cells localize more distantly from the endosteum, suggesting altered niche biology in the aged hematopoietic system.[34] Besides the discussed extrinsic mechanisms, the observed significantly lower percentage of older patients with normal trilineage hematopoiesis after ASCT might well be due to intrinsic stem cell changes. An potential cell intrinsic contributing factor might be telemore shortening, a mechanism implicated in aging and observed in a setting of chemotherapy treatment and ASCT.[35;36] However, assessment of telomere length after stem cell transplantation has shown inconsistent results regarding persistence of telomere shortening in patients.[37-40] Most probably a combination of both intrinsic and extrinsic factors is contributing to the age-related observations in ASCT. Further studies will be needed to elucidate underlying mechanisms in order to answer remaining questions.

Collectively, the presented data suggest that aging only has limited effects on $CD34^+$ human HSPCs as assessed by various *in vitro* assays and gene expression profiling. However, in the setting of ASCT we observed that advanced age strongly impairs long-term regeneration of peripheral blood cell counts. This could implicate that age-related changes of the hematopoietic system are most important in situations of chemotoxic and replicative stress.

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