Pegylated granulocyte colony-stimulating factor mobilizes CD34+ cells with different stem and progenitor subsets and distinct functional properties in comparison with unconjugated granulocyte colony-stimulating factor

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The online version of this article contains a supplemental appendix.

ABSTRACT

Background
Pegylated granulocyte colony-stimulating factor (G-CSF) has recently been introduced as a new compound for mobilization of CD34+ hematopoietic stem and progenitor cells. In this study, we compared the molecular and functional characteristics of CD34+ cells mobilized by pegylated G-CSF with those mobilized by unconjugated G-CSF.

Design and Methods
Gene expression of immunomagnetically enriched CD34+ cells from leukapheresis products of patients who were given pegylated-G-CSF or unconjugated G-CSF was analyzed using Affymetrix HG Focus microarrays and quantitative reverse transcriptase polymerase chain reaction. Flow cytometry and fluorescence activated cell sorting was conducted to assess the CD34+ subset composition and to obtain Lin-, CD34+, CD38- hematopoietic stem cells. Cell cycle assays and clonogenic assays were performed for functional corroboration.

Results
Pegylated G-CSF and unconjugated G-CSF mobilized CD34+ and hematopoietic stem cells with different molecular phenotypes and functional properties. The CD34+ cells mobilized by pegylated G-CSF had higher expression levels of genes indicative of early hematopoiesis, including *HOXA9*, *MEIS1* and *GATA3*. We found lower expression of genes characteristic of erythroid and later stages of myeloid differentiation and a lower functional burst-forming unit erythroid/colony-forming unit-granulocyte-macrophage ratio. Consistently, greater numbers of hematopoietic stem cells and common myeloid progenitors and fewer megakaryocyte-erthrocyte progenitors were found in the pegylated-G-CSF-mobilized CD34+ cells. Additionally, sorted pegylated-G-CSF-mobilized hematopoietic stem cells displayed higher expression of *HOXA9* in comparison to G-CSF-mobilized hematopoietic stem cells. In line with the gene expression data, CD34+ cells mobilized by pegylated G-CSF, as well as sorted hematopoietic stem cells, showed a significantly greater cell cycle activity.

Conclusions
Stimulation with pegylated-G-CSF or G-CSF results in different expression of key regulatory genes and different functional properties of mobilized hematopoietic stem cells as well as their progeny, a finding that might be relevant for the application of these cells in blood stem cell transplantation.

Key words: pegylated G-CSF, unconjugated G-CSF, autologous stem cell transplantation

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Introduction

Peripheral blood stem and progenitor cells are widely utilized for autologous and allogeneic grafts as they provide rapid and sustained hematologic reconstitution following high dose chemotherapy. In order to obtain a sufficient harvest, stem cells can be mobilized into the peripheral blood using cytokines, cytotoxic chemotherapy or a combination of both.[1] Currently, granulocyte colony-stimulating factor (G-CSF) is the most favored cytokine administered for peripheral blood stem cell mobilization because of its great efficacy and lack of serious toxicity. Recently, a modified form of recombinant human G-CSF has been introduced. This new compound is pegylated filgrastim (Peg-G-CSF) which has a 12-fold longer serum half-life than the unconjugated drug. Attachment of a polyethylene (glycol) moiety to recombinant human G-CSF reduces renal excretion and masks proteolytic cleavage sites resulting in elevated G-CSF serum levels for up to 14 days after a single injection.[2-4]

Following conventional cytotoxic chemotherapy, it has been observed that leukocyte recovery is more rapid and the occurrence of $CD34^+$ cells in the peripheral blood earlier after administration of Peg-G-CSF in comparison to unconjugated G-CSF.[5,6] The molecular causes underlying these different mobilization kinetics are unclear and the molecular characteristics and composition of Peg-G-CSF-mobilized stem and progenitor cells has not been studied so far. However, it has been previously demonstrated that Peg-G-CSF-mobilized $CD34^+$ cells and G-CSF-mobilized ones have different functional properties. Peg-G-CSF mobilization of $CD34^+$ cells resulted in enhanced expansion of tolerogenic antigen-presenting cells and augmentation of regulatory T-cell activity following transplantation and thus promoted tolerance.[7,8] In this study, we investigated the impact of Peg-G-CSF and unconjugated G-CSF at normalized cumulative doses on transcriptomal phenotype, subset composition and functional properties of $CD34^+$ cells and Lin^-, $CD34^+$, $CD38^-$ hematopoietic stem cells.

Design and Methods

Patients and cells

After informed consent, peripheral blood mononuclear cells were obtained by density centrifugation from 16 patients with multiple myeloma. There were no statistically significant differences with regard to age, gender, body weight, stage and subtype of disease, previous therapy or disease status between the two groups subsequently given Peg-G-CSF or G-CSF. The patients' characteristics are given in more detail in Table 1. Following induction therapy with a median of three (range, 2-6) cycles of 4×10 mg/m^2 idarubicin p.o. and 4×20 mg/m^2 dexamethasone p.o., all patients received a total dose of 4 g/m^2 cyclophosphamide administered on two consecutive days. Seven patients received a single dose of 6 mg Peg-G-CSF (median; range, 6-12 mg) 5 days (median; range, 4-6 days) after the end of chemotherapy. In the nine patients receiving unconjugated G-CSF a single subcutaneous injection was given once every day beginning 5 days (median; range, 4-8 days) after cytotoxic chemotherapy. The cumulative G-CSF dose administered was 6.2 mg (median; range, 4.3-15.4 mg). On a per kilogram basis the relative G-CSF dose was 109 μg (median; range, 76-197) in the Peg-G-CSF group and 111 μg (median; range, 54-256) in the G-CSF group. The dose on a per kilogram per day basis was 12 μg (median; range, 6-23) and 10 μg (median; range, 4-15) for the Peg-G-CSF group and G-CSF group, respectively. The collection of peripheral blood stem cells by apheresis was started in both groups of patients when a threshold concentration of 10 $CD34^+$ cells/μL was reached in the peripheral blood. $CD34^+$ cells were positively selected from apheresis products using the midiMACS immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) as described elsewhere.[9] Purities of $CD34^+$ cell preparations varied between 97.9% and 99.8%.

Table 1. Patients' characteristics.

	Peg-G-CSF Group	*G-CSF Group*
Age	55 years (43-66)	55 years (48-68)
Gender (male/female)	4 (44%)/ 5 (56%)	3 (43%)/ 4 (57%)
Body weight	70 kg (55-88)	70 kg (45-80)
IgG, A, LC	4 (44%), 5 (56%), 0 (0%)	5 (71%), 0 (0%), 2 (29%)
Stage I, II, III	0 (0%), 2 (22%), 7 (78%)	1 (14%), 0 (0%), 6 (86%)
Stage A, B	9 (100%), 0 (0%)	5 (71%) , 2 (29%)
Induction therapy (cycles)	3 (2-4) ID	3 (2-6) ID
Radiotherapy (total)	3 (33%)	0 (0%)
Extensive radiotherapy*	0 (0%)	0 (0%)
Remission status: PR, SD, PD	7 (78%), 1 (11%), 1 (11%)	4 (57%), 1 (14%), 2 (29%)

*LC, light chains; PR, partial response; SD, stable disease; PD, progressive disease; *means patients with radiotherapy of 7 or more vertebra and/or pelvis.*

RNA isolation, cRNA labeling and hybridization to microarrays

Total RNA (median: 6.0 μg; range, 1.5-35.5 μg) from isolated $CD34^+$ cells was used to generate biotin-labeled cRNA (median: 27.2 μg; range, 4.8-105.3 μg) by means of the Enzo BioArray HighYield RNA Transcript Labeling Kit (Affymetrix Ltd, UK). Quality control of RNA and cRNA was performed using a bioanalyzer (Agilent 2001 Biosizing, Agilent Technologies). Following fragmentation, labeled cRNA from samples of each individual patient was hybridized to Affymetrix HG-Focus GeneChips (one array per patient's sample) covering 8793 genes and stained according to the manufacturer's instructions.

Quantification, normalization, and statistical analysis

Details on quality control, normalization and data analysis, are given in the *Online Supplementary Data*.[10,11]

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

RNA expression data were corroborated by real-time RT-PCR using the ABI PRISM® 7900HT Sequence Detection System Instrument (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany). Total RNA was reverse-transcribed as described previously.[11] PCR was performed in a MicroAmp Optical 96-well Reaction Plate (Applera) according to the instructions of the manufacturer using commercially available assays-on-demand. *GAPDH* mRNA served as an external control for relative quantification. Relative gene expression levels are presented as the difference of C_T values of the target gene and *GAPDH* (ΔC_T). For RT-PCR analysis of lin$^-$CD34$^+$ CD38$^-$ subsets, total RNA was isolated using the RNeasy micro kit (Qiagen, Hilden, Germany) adding 20 ng of bacterial rRNA as a carrier according to the manufacturer's instructions.

Cell cycle assays

Cell cycle analysis using BrdU and 7-amino-actinomycin D (7-AAD) staining was performed as described previously.[12] Student's t-test was used to assess statistically significant differences regarding cell cycle phases between the two groups given Peg-G-CSF or G-CSF ($p<0.05$).

Immunofluorescence-based cell sorting

Flow cytometric analysis of CD34$^+$ subsets and sorting of Lin$^-$, CD34$^+$, CD38$^-$ hematopoietic stem cells was performed as described previously.[13] Cells were sorted and analyzed using a double laser (488 nm/350 nm Enterprise II +647 Spectrum) high-speed cell sorter (MoFlo MLS, Cytomation).

Semisolid clonogenic assays

Mononuclear cells and purified CD34$^+$ cells were seeded in semisolid ready-to-use methylcellulose growth medium (MethoCult H4436, StemCell Technology, Vancouver, Canada) at concentrations ranging between 2×10^4 and 5×10^4 MNC/mL and 5×10^2 and 1×10^3 CD34$^+$ cells/mL as described.[14] Colony numbers (CFU-GM, BFU-E) were counted after 2 weeks. Each experiment was performed in duplicate. The Student's t-test was used to detect statistically significant differences ($p<0.05$).

Results

In this study, we compared gene expression patterns, subset composition and functional properties of peripheral blood-derived CD34$^+$ cells and highly purified hematopoietic stem cells obtained from patients with multiple myeloma following mobilization with cyclophosphamide and stimulation with either Peg-G-CSF or G-CSF. Approaching the period of leukopenia following a median time of 5 days after the end of chemotherapy, seven patients were given a single injection of Peg-G-CSF while nine patients received G-CSF on a daily basis resulting in an equal cumulative dose. Looking at a total of 8793 genes contained within the array we found that 339 genes were differentially expressed with a q-value below 5% and a fold change of at least 1.2. Comparing the two groups of patients, 222 genes had a higher expression level within the Peg-G-CSF-mobilized CD34$^+$ cells and 117 genes had a lower one in comparison to the G-CSF-mobilized CD34$^+$ cells. Hierarchical cluster analysis on the basis of the differentially expressed genes reflected the distinct expression pattern of G-CSF and Peg-G-CSF-mobilized cells and demonstrated the homogeneity of the cell samples of each group (*Online Supplementary Figure 1*). Microarray gene expression analysis was performed in conformity with the latest consensus guidelines.[15] The complete gene expression data can be accessed online via *http://www.ncbi.nlm.nih.gov/geo/*; accession no. GSE4688. A selection of differentially expressed genes is assigned to functional groups and shown in *Online Supplementary Table 1*.

Peg-G-CSF-mobilized peripheral blood stem cells show a gene expression pattern characteristic of immature progenitors

In Peg-G-CSF-mobilized CD34$^+$ cells, we found a gene expression pattern reflecting a developmentally earlier progenitor cell type. Accordingly, in Peg-G-CSF-mobilized CD34$^+$ cells significantly greater levels of the *HOX* family of homeobox genes such as *HOXA9*, *HOXA10* and their positive regulator *MLL* as well as co-factor myeloid ecotropic viral integration site 1 homolog (*MEIS1*) were observed. On the other hand, genes associated with erythroid differentiation and late stages of myeloid matura-

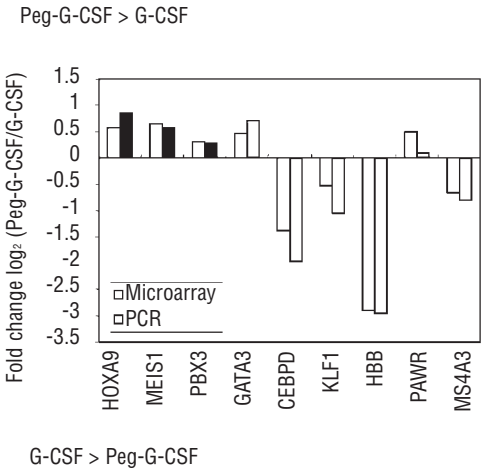

Figure 1. Confirmation of microarray expression data by RT-PCR. Fold changes determined by microarray analysis are represented by white columns, fold changes determined using RT-PCR are shown by gray columns. Fold changes >0 indicate higher expression following Peg-G-CSF mobilization, fold changes <0 indicate higher expression after G-CSF stimulation. The data on the y axis are plotted as log base 2.

tion along the granulocytic lineage were expressed at 2- to 16-fold lower levels in Peg-G-CSF-mobilized CD34+ cells compared to G-CSF-mobilized ones. These genes included β-globin and the global erythroid regulator and transcription factor Kruppel-like factor 1 (*KLF1*),[16] defensins α 1,3 and 4 and C/EBP β and δ as well as matrix metalloproteinases 8 and 9. In turn, *PAWR* (Par-4 receptor), a regulator of Wilm's tumor gene 1 (*WT1*)[17] and *HOXA5*[18] both of which are inhibitors of erythroid differentiation, showed a significantly higher expression level in Peg-G-CSF-mobilized cells.

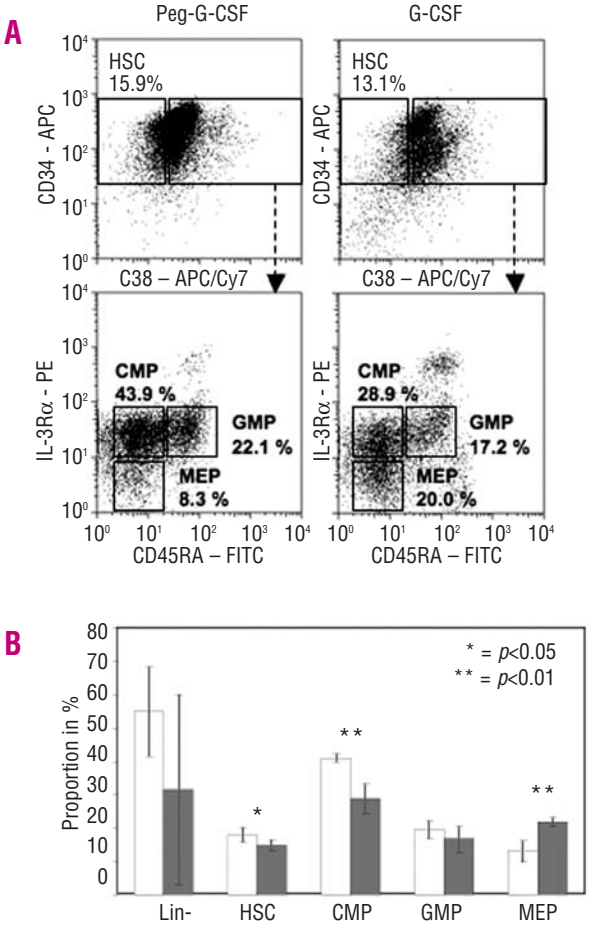

Figure 2. Different patterns of hematopoietic stem and progenitor cells in the peripheral blood of patients stimulated with either Peg-G-CSF (left) or G-CSF (right). A. Immunomagnetic selection of CD34+ cells followed by multicolor flow cytometry was utilized to analyze hematopoietic stem and progenitor cell subsets. After gating on viable cells and lineage-depletion subfractions of hematopoietic stem cells (Lin-, CD34+, CD38-), common myeloid progenitors (Lin-, CD34+, CD38+, IL-3Ra+, CD45RA-), granulocyte monocyte progenitors (Lin-, CD34+, CD38+, IL-3Ra+, CD45RA+), and megakaryocyte erythrocyte progenitors (Lin-, CD34+, CD38+, IL-3Ra-, CD45RA-) were determined. Percentages related to Lin- cells are indicated. Dot plots of one representative experiment out of five are shown. B. Bar chart displaying medians of all experiments performed. White columns represent Peg-G-CSF-mobilized cells and gray columns represent G-CSF-mobilized cells. Standard deviations are indicated. The percentage for Lin- cells is related to total CD34+ cells while the percentages for hematopoietic stem cells, common myeloid progenitors, granulocyte monocyte progenitors and megakaryocyte erythrocyte progenitors are related to Lin-, CD34+ cells.

Compared to G-CSF stimulation, Peg-G-CSF mobilizes a greater proportion of hematopoietic stem cells and common myeloid progenitors but fewer megakaryocyte-erythrocyte progenitors

To address the question of whether the distinct expression patterns could result from a different progenitor subset composition (hematopoietic stem cells, common myeloid progenitors, granulocyte monocyte progenitors, megakaryocyte erythrocyte progenitors) in the peripheral blood after stimulation by either G-CSF or Peg-G-CSF, we examined five G-CSF-mobilized and five Peg-G-CSF-mobilized CD34+ cell samples with regard to their subset composition. Peg-G-CSF-mobilized samples contained a greater proportion of hematopoietic stem cells (17.8%; SD: 2.1% vs. 14.1%; SD: 1.8%; p=0.038) and a higher fraction of common myeloid progenitors (40.9%; SD: 1.1% vs. 28.7%; SD: 4.4%; p=0.008) compared with G-CSF-mobilized samples (Figure 2). In contrast, we found a significantly lower prportion of megakaryocyte erythrocyte progenitors (12.9%; SD:3.0% vs. 21.8%; SD:1.4%; p=0.005) in Peg-G-CSF-mobilized cells. These data demonstrate that the subset composition of Peg-G-CSF-mobilized cells is different from that of G-CSF-mobilized cells and partially explains the more immature transcriptional profile with less proneness to erythroid differentiation.

Peg-G-CSF-mobilized PBSC favor granulocytic over erythroid colony formation

We hypothesized that the observed higher expression levels of genes important for early myeloid progenitors and, correspondingly, the greater proportion of common myeloid progenitors, combined with lower expression of erythropoiesis-related genes and the smaller proportion of megakaryocyte erythrocyte progenitors in Peg-G-CSF-mobilized CD34+ cells are relevant for the colony-forming potential of these cells. To address this question, we utilized semisolid methylcellulose assays to determine clonogenic growth. Mononuclear cells as well as purified CD34+ cells obtained from patients who had received Peg-G-CSF were associated with significantly lower mean BFU-E/CFU-GM ratios than CD34+ cells from patients given G-CSF (p=0.038 and p=0.016, respectively) (Figure 3A and B).

Peg-G-CSF-mobilized peripheral blood stem cells express higher levels of proliferation-associated genes

We found seven differentially expressed genes involved in cell cycle regulation (*Online Supplementary Table 1*). Six of them are known to drive cell cycle progression and one gene causes G_0/G_1 cell cycle arrest.[19-23] The cell cycle promoting genes including cyclins, kinases, and small G-protein superfamily members were expressed at 1.3- to 1.7-fold higher levels in Peg-G-CSF-mobilized CD34+ cells compared to their G-CSF-mobilized counterparts. On the other hand, the expression level of the hematopoietic cell-specific inhibitory cell cycle modulator membrane spanning four protein family group member A3 (*MS4A3*)[20] was

1.5-fold lower in Peg-G-CSF-mobilized CD34$^+$ cells. We did, however, find a higher expression level of cyclin D3 in G-CSF-mobilized cells. This might be due to lineage-dependent overexpression, as cyclin D3 is upregulated through erythroid-megakaryocytic differentiation.[22] In summary, the gene expression pattern suggests higher cell cycle activity in Peg-G-CSF-mobilized cells.

Peg-G-CSF-mobilized peripheral blood stem cells have a greater cycling activity

In order to examine whether the higher expression levels of proliferation-associated genes in Peg-G-CSF-mobilized cells are functionally relevant, we performed cell cycle analyses. In line with the gene expression data we saw a significantly greater proportion of actively cycling CD34$^+$ cells (S-phase) in the samples from patients given Peg-G-CSF than in those given G-CSF. The percentage of Peg-G-CSF-mobilized CD34$^+$ cells in $G_0/_1$ phase was significantly smaller (Figure 4). Checking for functional differences also in hematopoietic stem cells (Figure 5B,C) we found a greater proportion of Lin$^-$, CD34$^+$, CD38$^-$ hematopoietic stem cells in G_2/M phase (p=0.05) among Peg-G-CSF-mobilized cells (median 25.7%; range, 21.9-29.4%; SD 5.3) than in G-CSF-mobilized cells (median 9.4%; range, 8.3-10.5%; SD 1.6). A higher percentage of hematopoietic stem cells mobilized by G-CSF was in $G_0/_1$ phase (median 90%; range, 88.8-91.1%; SD 1.6) when compared to Peg-G-CSF-mobilized cells (median 72.8%; range, 69.0-76.5%; SD 5.3; p=0.05). On average, twice as many hematopoietic stem cells mobilized by Peg-G-CSF were in S-phase (median 0.6%; range, 0.5-0.7%; SD 0.1) compared to those mobilized by G-CSF (median 0.3%; range, 0.1-0.5%; SD 0.3). However, this difference was not statistically significant (p=0.3). In conclusion, Peg-G-CSF fosters cell cycle activity in CD34$^+$ cells as well as in Lin$^-$, CD34$^+$, CD38$^-$ cells in accordance with the gene expression data.

Elevated expression levels of HOXA9 and GATA3 in Peg-G-CSF-mobilized hematopoietic stem cells

After having found that the distinct gene expression profiles of Peg-G-CSF- and G-CSF-mobilized CD34$^+$ cells are also reflected by differences in subset composition and functional properties, we next addressed the question of whether differential gene expression in CD34$^+$ cells might also be a result of Peg-G-CSF- versus G-CSF-induced transcriptional changes in early hematopoietic stem cells. We sorted Lin$^-$, CD34$^+$, CD38$^-$ hematopoietic stem cells from seven G-CSF-mobilized and seven Peg-G-CSF-mobilized mononuclear cell samples and performed RT-PCR analysis for β-globin, *KLF1*, *GATA3* and *HOXA9*. Strikingly, we found a significantly higher expression level of *HOXA9* (p=0.02) in Peg-G-CSF-mobilized hematopoietic stem cells in comparison with G-CSF-mobilized ones. For *GATA3* there was a clear trend towards higher expression in Peg-G-CSF-mobilized, hematopoietic stem cells, but the differ-

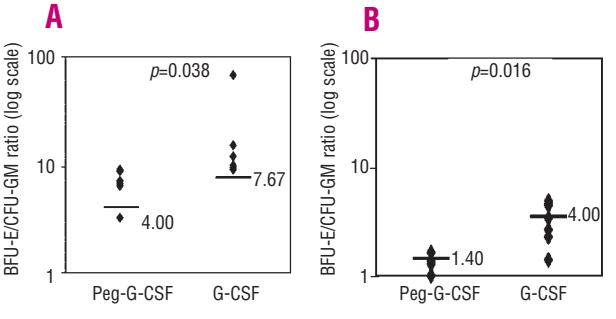

Figure 3. Clonogenic assays of mononuclear cells (A) and purified CD34$^+$ cells (B) of patients mobilized by either Peg-G-CSF (left) or G-CSF (right). Mononuclear cells from apheresis products of patients mobilized with either Peg-G-CSF (n=7) or G-CSF (n=7) were seeded in semisolid ready-to-use methylcellulose growth medium containing stem cell factor, granulocyte-monocyte, colony-stimulating factor, interleukin-3, interleukin-6, and erythropoietin (A). To corroborate these results with purified CD34$^+$ cells, seven G-CSF-mobilized and four Peg-G-CSF-mobilized samples were immunomagnetically selected and plated as described for the monuclear cells (B). Colony numbers (colony-forming units granulocyte/macrophage, CFU-GM; burst-forming units erythrocyte BFU-E) were scored after 2 weeks. Diamonds represent the BFU-E/CFU-GM ratio of each clonogenic assay and bars represent the respective median ratio. Medians of the BFU-E/CFU-GM ratio are given in the diagram. The p value is indicated.

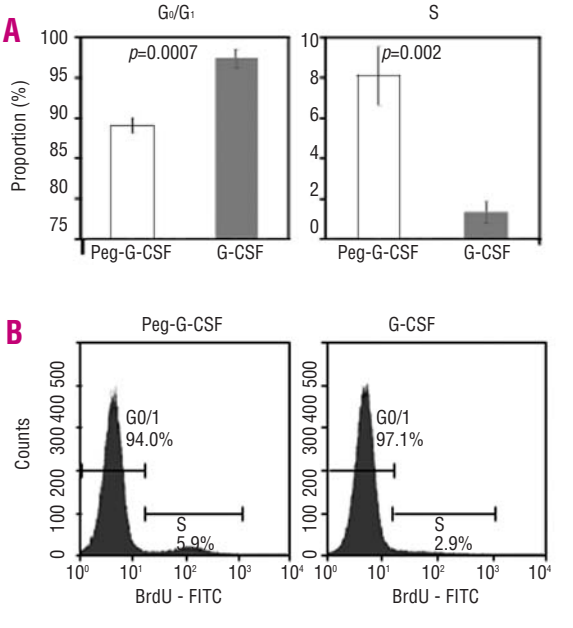

Figure 4. A. Cell cycle assays of hematopoietic stem and progenitor cells in the peripheral blood of patients mobilized by either Peg-G-CSF (white columns) or G-CSF (grey columns). Cell cycle analysis using BrdU and 7 amino-actinomycin D staining was performed after 3 hours of cell culture in RPMI 1640 media containing fetal calf serum, interleukin-3, interleukin-6 and stem cell factor and labeling with BrdU. After permeabilization and staining with fluorochrome-conjugated anti-BrdU antibodies and 7-amino-actinomycin D, flow cytometric analysis was performed. Student's t test was used to assess statistically significant differences regarding cell cycle phases between the two groups (p<0.05). The G_0/G_1 phase of the cell cycle is displayed, on the left side, the S phase on the right. B. Histogram plots of one representative experiment of four using Peg-G-CSF- (left) and G-CSF-mobilized CD34$^+$ cells (right) are shown. The percentages of cells in the G_0/G_1 phase and S phase are indicated.

ence was not statistically significant (Figure 5A). In contrast to the situation in the overall CD34$^+$ cell population, the β-globin gene and *KLF1* were not differentially expressed in the Lin$^-$, CD34$^+$, CD38$^-$ hematopoietic stem cell subset suggesting that they might be affected at later developmental stages. Taken together, these findings demonstrate that, compared to G-CSF stimulation, stimulation with Peg-G-CSF not only leads to mobilization of CD34$^+$ cells with distinct subset compositions but also to different expression levels of transcriptional key regulators in Lin$^-$, CD34$^+$, CD38$^-$ hematopoietic stem cells.

Discussion

Here, we compared the effects of stimulation with Peg-G-CSF and unconjugated G-CSF on mobilized hematopoietic stem cells as well as their progeny. In Peg-G-CSF-mobilized CD34$^+$ cells, we found a gene expression pattern reflecting a developmentally earlier progenitor cell type, including the *HOX* family of homeobox genes, *MEIS1* and *MLL*. These genes are expressed at high levels in hematopoietic stem and immature progenitor cells, thus suggesting a greater proportion of early, uncommitted stem and immature progenitor cells within the CD34$^+$ cell population.[24-29] Differences in the expression levels of these genes between Peg-G-CSF and G-CSF-mobilized cells were relatively small (*see Online Supplementary Table 1*). Nevertheless, we and others have previously shown that even modest differences in the expression levels of transcription factors involved in hematopoiesis can lead to altered gene expression with a substantial influence on stem cell differentiation and function.[30-33]

In contrast, genes associated with erythroid and later stages of myeloid differentiation were expressed at lower levels. Of these genes, defensins α 1, 3 and 4 play a pivotal role in neutrophil defense mechanisms[34,35] and transcription factors C/EBP β and δ are crucially involved in the maturation of cells commited to the myeloid lineage.[36-38] Accordingly, matrix metalloproteinases 8 (*MMP8*) and 9 (*MMP9*) were expressed at lower levels. *MMP8* is a gene under C/EBP family control and expressed late in the myeloid maturation pathway.[38] Its family member *MMP9* promotes differentiation at an earlier stage. *MMP9* acts on hematopoietic stem cells via release of soluble kit ligand and induces transition from a quiescent to a proliferative stage, thereby favoring differentiation.[39] In line with the gene expression profile, analysis of the CD34$^+$ subset composition of Peg-G-CSF-mobilized cells showed significantly greater proportions of hematopoietic stem cells and common myeloid progenitors, and a lower proportion of megakaryocyte erythrocyte progenitors. For functional corroboration of these findings we performed clonogenic assays and found a significantly lower BFU-E/CFU-GM ratio after plating of Peg-G-CSF-mobilized CD34$^+$ cells compared to cells mobilized by unconjugated G-CSF. This finding is indicative of a higher proportion of myeloid progenitor cells and a smaller proportion of progenitor cells committed to the erythroid lineage within the Peg-G-CSF-mobilized CD34$^+$ cells and demonstrates the functional relevance of the observed differential gene expression profiles and progenitor subset composition. Higher levels of expression of genes functionally important for hematopoietic stem cells were found not only in the CD34$^+$ cells but also in highly purified hematopoietic stem cells after mobilization with Peg-G-CSF, suggesting enhanced long-term repopulating ability.[28,40] This might explain the results of a recent clinical trial in which the authors found significantly greater leukocyte, reticulocyte

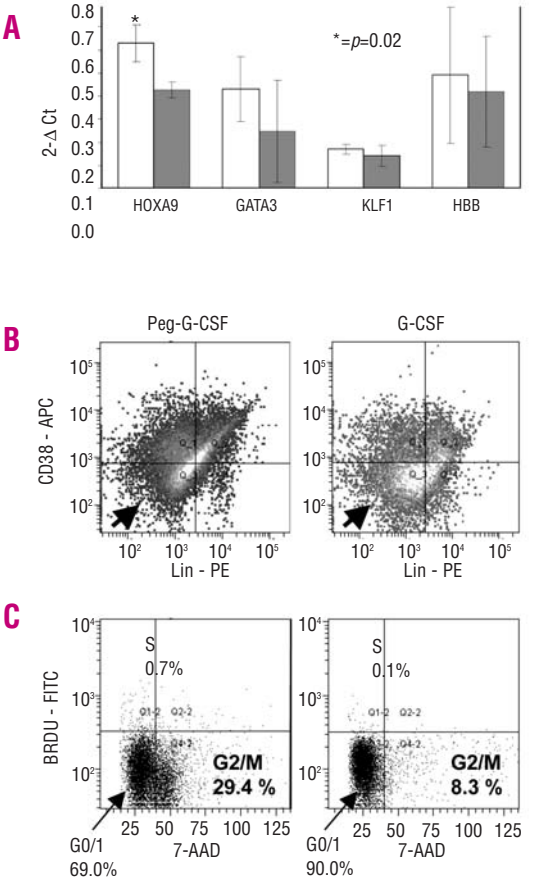

Figure 5. Quantitative real-time RT-PCR for *HOXA9* and *GATA3* mRNA and cell cycle assays of hematopoietic stem cells mobilized by either Peg-G-CSF (left) or G-CSF (right). A. Highly enriched Lin$^-$, CD34$^+$, CD38$^-$ hematopoietic stem cells mobilized by either Peg-G-CSF or G-CSF were subjected to two independent quantitative real-time RT-PCR series for *HOXA9* and *GATA3*. ΔC_T values were calculated by subtracting the C_T value of the reference gene from the C_T value of target genes. The data on the y axis are plotted as 2-CT. Peg-G-CSF-mobilized hematopoietic stem cells are represented by white columns, G-CSF-mobilized cells are shown by gray columns. The higher the 2-CT value of the target gene, the greater the expression level of this gene. Cell cycle analysis using BrdU and 7 amino-actinomycin D staining was performed after 3 hours of cell culture in RPMI 1640 media containing fetal calf serum, interleukin-6, interleukin-3 and stem cell factor and labeling with BrdU. After permeabilization and staining with fluorochrome-conjugated anti-BrdU antibodies and 7-amino-actinomycin D, flow cytometric analysis was performed. B. To specifically analyze the hematopoietic stem cell subset, a gate was set on Lin$^-$, CD34$^+$, CD38$^-$ cells (black arrow) utilizing multi-color flow cytometry. C. One representative experiment out of three for cell cycle analysis of Lin$^-$, CD34$^+$, CD38$^-$ cells is shown.

and platelet counts on day 100 after initial engraftment following transplantation of Peg-G-CSF-mobilized autografts compared to grafts mobilized by unconjugated G-CSF.[41] Of interest, the number of Peg-G-CSF mobilized $CD34^+$ cells transplanted was even smaller than the number of G-CSF-mobilized cells (p=0.0575). Hence, it was assumed that different biological functions of Peg-G-CSF-mobilized cells may have accounted for these observations.[41] Looking at the engraftment kinetics of the patients whose mobilized stem and progenitor cells were analyzed here in our study, we also found significantly higher white blood cell count on day 100 post-transplantation in patients given Peg-G-CSF. At that time the median white cell count was 6.0×10^3/μL after transplantation of Peg-G-CSF mobilized cells compared to 3.3×10^3/μL after transplantation of G-CSF mobilized cells (p=0.03). Platelet counts and hemoglobin levels were also higher, although not statistically significantly so. However, due to the small sample size, these observations need to be substantiated in larger, randomized clinical trials. Of interest, mobilization of greater proportions of hematopoietic stem cells with superior long-term repopulating capacity have also been described for other mobilizing agents such as CXCR4 antagonists and GROβ, either used as single agents or in combination with G-CSF.[42-44] Apart from the superior long-term repopulating capacity, these cells also show accelerated short-term hematopoietic recovery. At first glance, this finding seems surprising as one would expect that more mature hematopoietic progenitor cells rather than immature primitive hematopoietic stem cells would contribute to accelerated short-term hematopoietic recovery.[45] However, other studies also found that not only long-term marrow recovery but also rapid short-term recovery depends on hematopoietic stem cells.[46-48]

In this context, it may be asked whether platelet recovery is impaired after transplantation of Peg-G-CSF-mobilized cells due to a significantly smaller proportion of megakaryocyte erythrocyte progenitors. Looking at the engraftment kinetics after transplantation of the grafts analyzed here, neither short-term nor long-term thrombocytopenia was observed. However, this finding also needs to be confirmed in larger randomized clinical trials. Peg-G-CSF-mobilized cells had a higher cell cycle activity compared to their G-CSF mobilized counterparts, consistent with the gene expression profile. Initially, this finding was puzzling since Peg-G-CSF-mobilized $CD34^+$ cells contained a greater proportion of hematopoietic stem cells, which are generally considered quiescent and even these Peg-G-CSF-mobilized Lin^-, $CD34^+$, $CD38^-$ cells showed a greater proportion of cells in the S and G_2/M phases of the cell cycle. However, Shojaei *et al*. have recently demonstrated a higher reconstitution ability of hematopoietic stem cells with increased cycling frequency,[49] which is in line with our data.

Searching for the underlying mechanism that may account for the different transcriptional and functional phenotypes of Peg-G-CSF-mobilized cells, it has been previously shown in a murine G-CSF receptor knock-out model that Peg-G-CSF and G-CSF exert their pharmacological effects via the same G-CSF receptor.[50] Thus, the different effects of G-CSF and Peg-G-CSF do not seem to be related to activation of different receptors. Neither absolute $CD34^+$ cell count/μL peripheral blood nor total $CD34^+$ cell yield seems to be differentially affected by Peg-G-CSF and G-CSF as we found no significant differences after mobilization with Peg-G-CSF and G-CSF, in line with data reported previously for steady state- and cytotoxic mobilization.[51,52] Of note, $CD34^+$ subset composition after Peg-G-CSF and G-CSF mobilization described here was not addressed in those studies referenced above.

Interestingly, in a recent randomized clinical trial the effect of continuous intravenous administration vs. daily single subcutaneous doses of G-CSF on $CD34^+$ cell mobilization was examined.[53] The authors found that $CD34^+$ cell peak concentrations were reached 2 days earlier following continuous intravenous G-CSF administration compared to daily subcutaneous injections. These findings and the mobilization kinetics observed following the administration of Peg-G-CSF suggest that the time-course of stimulation (pulsatile versus continuous), rather than a dose-related mechanism, might account for the distinct effects of Peg-G-CSF and G-CSF on stem and progenitor cells.

In conclusion, stimulation with Peg-G-CSF versus G-CSF, despite the same active compound, leads to altered gene expression of key regulatory genes and different functional properties of mobilized hematopoietic stem cells as well as their progeny. This, combined with a different subset composition of Peg-G-CSF-mobilized $CD34^+$ stem and progenitor cells, may account for the distinct functional properties and differentiation patterns observed. These findings might explain the recently reported different clinical properties and engraftment kinetics of Peg-G-CSF-mobilized cells after autologous transplantation.

Authorship and Disclosures

IB: designed the study, performed experiments, analyzed data, wrote the manuscript; US designed the study, performed experiments, analyzed data, wrote the manuscript; JCF performed experiments, analyzed data, reviewed the manuscript; AC performed experiments, reviewed the manuscript; GK designed the study, provided biological samples, reviewed the manuscript; SR performed experiments; RF provided biological samples; MR performed bioinformatical analysis; SP performed experiments: AVH: head of bioinformatics department, reviewed the mansucript; PW reviewed the manuscript; DGT designed the study, reviewed the manuscript; RH designed the study, reviewed the manuscript; RK designed the study, analyzed data, reviewed the manuscript. The authors reported no potential conflicts of interest.

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