Heparan sulfate mimetics can efficiently mobilize long-term hematopoietic stem cells

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ABSTRACT

Background

Although mobilization of hematopoietic stem cells and hematopoietic progenitor cells can be achieved with a combination of granulocyte colony-stimulating factor and plerixafor (AMD3100), improving approaches for hematopoietic progenitor cell mobilization is clinically important.

Design and Methods

Heparan sulfate proteoglycans are ubiquitous macromolecules associated with the extracellular matrix that regulates biology of hematopoietic stem cells. We studied the effects of a new family of synthetic oligosaccharides mimicking heparan sulfate on hematopoietic stem cell mobilization. These oligosaccharides were administered intravenously alone or in combination with granulocyte colony-stimulating factor and/or AMD3100 in mice. Mobilized hematopoietic cells were counted and phenotyped at different times and the ability of mobilized hematopoietic stem cells to reconstitute long-term hematopoiesis was determined by competitive transplantation into syngenic lethally irradiated mice followed by secondary transplantation.

Results

Mimetics of heparan sulfate induced rapid mobilization of B-lymphocytes, T-lymphocytes, hematopoietic stem cells and hematopoietic progenitor cells. They increased the mobilization of hematopoietic stem cells and hematopoietic progenitor cells more than 3-fold when added to the granulocyte colony-stimulating factor/AMD3100 association. Hematopoietic stem cells mobilized by mimetics of heparan sulfate or by the granulocyte colony-stimulating factor/AMD3100/mimetics association were as effective as hematopoietic stem cells mobilized by the granulocyte colony-stimulating factor/AMD3100 association for primary and secondary hematopoietic reconstitution of lethally irradiated mice.

Conclusions

This new family of mobilizing agents could alone or in combination with granulocyte colony-stimulating factor and/or AMD3100 mobilize a high number of hematopoietic stem cells that were able to maintain long-term hematopoiesis. These results strengthen the role of heparan sulfates in the retention of hematopoietic stem cells in bone marrow and support the use of small glyco-drugs based on heparan sulfate in combination with granulocyte colony-stimulating factor and AMD3100 to improve high stem cell mobilization, particularly in a prospect of use in human therapeutics.

Key words: hematopoietic stem cell mobilization, heparan sulfate, hematopoietic reconstitution, VEGF, SDF-1α.

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The online version of this article has a Supplementary Appendix.

Introduction

In adult mammals, a contingent of hematopoietic stem cells (HSC) present in the bone marrow (BM) gives rise to all mature blood cells through the process of hematopoiesis. These cells are present in very small number in BM and require a particular microenvironment to self-renew and/or give rise to hematopoietic progenitor cells (HPC). HSC are located within specialized niches in the BM[1,2] consisting of hematopoietic and non-hematopoietic cells and the extracellular matrix (ECM).[3-5] This microenvironment drives the fate of HSC by regulating their proliferation, quiescence and/or determination. Although tightly bound to their niches, HSC can traffic between BM, peripheral blood (PB) and secondary organs in response to endogenous or exogenous stresses, a process called mobilization.[6-8] Conversely, injection of HSC into lethally irradiated mice results in migration of the HSC from the bloodstream to the BM, followed by homing and engraftment of the cells in the BM. Cell mobilization from the BM to the PB has become an important source of hematopoietic stem and progenitor cells (HSPC) for hematopoietic stem cell transplantation and the optimal method to mobilize HSPC remains a field of intense research. Three cross-talking pathways mainly regulate mobilization: the first one involves the chemokine receptor CXCR4 and its ligand stromal cell derived factor 1 (SDF-1), the second one involves the $\alpha4\beta1$ integrin VLA-4 and its ligand VCAM-1 and the third one involves interactions between HSC and various ECM components.[9,10] Granulocyte colony-stimulating factor (G-CSF), the most common therapeutic agent used to mobilize HSC and HPC for transplantation in patients after myeloablative treatment,[11,12] essentially acts through disruption of the SDF-1/CXCR4 axis.[12-14] However, broad inter-individual variability of mobilization efficacy exists and poor HSPC mobilization in response to G-CSF is observed in patients who have previously been treated with multiple rounds of intensive chemotherapy or in patients with genetic disorders, such as Fanconi's anemia.[13] To enhance HSPC mobilization, therapeutic agents such as chemotherapeutic drugs (cyclophosphamide and 5-fluorouracil), cytokines (such as interleukin-1[15] or interleukin-8)[16] chemokines (such as MIP-1α[17] or SDF-1)[18,19] or synthesized small molecules (such as AMD3100)[20] have been used alone or in combination with G-CSF. This long array of mobilizing agents has been used, clinically and experimentally in animal models, to induce HSC mobilization[21] and transplantation of PB containing a high number of mobilized HSC has become a standard in the treatment of many disorders.[22-24] Currently the most promising mobilizing agent is the bicyclam molecule AMD3100 which specifically and reversibly blocks SDF-1 binding to CXCR4 and induces rapid mobilization of mouse and human HSPC.[14] AMD3100 was shown to be able to rapidly mobilize HSPC from the BM into the PB of mice deficient in CD26 (a protease present at the surface of HSC which can cleave CXCL12), not responding to the effect of G-CSF on mobilization.[25]

The ECM plays an important role in the retention of HSC in the BM and tightly regulates HSC functions. Heparan sulfate proteoglycans are a major constituent of the ECM[26] as their polysaccharide component, heparan sulfate, a member of the glycosaminoglycans family, can bind a wide variety of biological mediators such as growth factors, chemokines, interleukins and proteolytic enzymes and can regulate their bioavailability and functionality.[27-29] Heparan sulfate proteoglycans are important actors in the HSC niches in BM because they bind cytokines and present them to stromal cells and HSC.[30] Cleavage of ECM proteins by metalloproteases is involved in HSC mobilization[10,31] and sulfate glycans, which rapidly mobilize HPC,[32] can compete with HPC for binding the ECM components. HSC mobilization is also associated with an increased concentration of SDF-1 in blood and a decreased concentration of SDF-1 in the BM,[33] likely due to enzymatic cleavage and decreased gene expression.

In this study, we investigated the effects of a new class of oligosaccharides, heparan sulfate mimetics, on the mobilization of HSC, alone or in combination with AMD3100 and G-CSF conditioning.

Design and Methods

Animals

Mice of the strain C57BL/6J were obtained from the *Centre de Recherche et d'Elevage Janvier* (France). The animals were cared for in accordance with French Government procedures (*Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture,* France).

Mobilization of white blood cells by granulocyte colony-stimulating factor, AMD3100, EP80006 and EP80031

EP80006 and EP80031 are, respectively, synthetic hexa- and octo-saccharides (Figure 1A) produced by Endotis Pharma. These oligosaccharides were shown to be free of endotoxins (Lonza, Verviers, Belgium). G-CSF (Neupogen, Amgen), AMD3100 (Sigma-Aldrich), EP80006 an EP80031 (Endotis Pharma, EP) were supplied as isotonic aqueous solutions. G-CSF was administered subcutaneously twice a day for 2 or 4 days and the morning of the third or fifth day at the dose of 2.5 μg for each mouse. AMD3100 was administered subcutaneously in a single injection at the dose of 5 mg/kg. EP80031 and EP80006 were administered intravenously in a single injection at the dose of 15 mg/kg. Blood samples were collected 30 min, 1, 3 and 5 h after the last injection of G-CSF or the injection of AMD3100 and/or EP80031 and EP80006. Peripheral blood leukocytes (PBL) were counted with an automatic hematology analyzer (Abacus Junior Vet; Diatron, Vienna, Austria) and then labeled with a combination of fluorescent antibodies to characterize the mobilized HSPC.

Four combinations of compounds were used to study white blood cell mobilization: (i) AMD3100 + EP80031, (ii) G-CSF + EP80031, (iii) G-CSF + AMD3100 and (iv) G-CSF + AMD3100 + EP80031. PBL samples were collected 30 min, 1, 3 and 5 h after the last injection of AMD3100 or EP80031.

Flow cytometry

Peripheral blood cells were collected from the retro-orbital vein and BM cells were flushed from both tibiae and femora of C57Bl/6Ly5.2 (CD45.2) or C57Bl/6Ly5.1 (CD45.1) mice. PB or BM cells were then treated with a 0.75% NH_4Cl solution (Sigma-Aldrich, St Louis, MO, USA) to lyse red blood cells. Blood leukocytes and BM cells were stained with appropriate fluorescent antibodies as described in the *Online Supplementary Design and Methods*.

Quantification of vascular endothelial growth factor and stromal cell-derived factor 1

Mouse vascular endothelial growth factor (VEGF) and SDF-1α plasma immunoassays were performed according to the manufacturer's recommendations (Quantikine assays, R&D Systems).

Details of the VEGF and SDF-1α immunoassays are provided in the *Online Supplementary Design and Methods.*

Colony-forming unit assay

The colony-forming unit (CFU) assay (MethoCult 03434, StemCell Technologies) was performed according to the manufacturer's instructions. Details of the CFU assay on blood samples are provided in the *Online Supplementary Design and Methods*.

Competitive assay: transplantation of mobilized KLS cells

C57Bl/6 CD45.2 recipient mice that had received lethal irradiation with 11 Gy were transplanted with 15,000 PB KLS cells mobilized with G-CSF, G-CSF + EP80031, G-CSF + AMD3100 or G-CSF + AMD3100 + EP80031 from C57Bl/6 CD45.1 donor mice in competition with 3000 KLS competitor cells isolated from the BM of non-conditioned C57Bl/6 CD45.2 mice.

Engraftment levels were assessed by the determination of the proportion, in PB and BM, of CD45.1 donor cells compared to CD45.2 competitor cells 4 months after transplantation (long-term engraftment). White blood cells (WBC) were labeled in order to assess the proportion of leukocyte populations and concentrations in PB and BM.

Secondary transplantation

Four months after competitive transplantation, 500,000 BM cells derived from competitive transplantation with PB KLS mobilized by G-CSF + AMD3100 or G-CSF + AMD3100 + EP80031 were transplanted into C57BL/6 CD45.2 lethally irradiated mice. Engraftment levels and WBC labeling were performed on blood leukocytes and BM cells 4 months after transplantation.

Limited dilution competitive assay: transplantation of mobilized leukocytes

C57Bl/6 CD45.1 donor mice were treated with G-CSF, G-CSF + EP80031, G-CSF + AMD3100 or G-CSF + AMD3100 + EP80031. The blood leukocytes of each group of treated mice were pooled and injected in competition with total BM CD45.2$^+$ cells into lethally irradiated C57Bl/6 CD45.2 recipient mice with a ratio of donor (CD45.1) blood cells to competitor (CD45.2) BM cells defined by the number of mononuclear blood cells of X donor mice to 5×10^5 competitor BM cells.[14] Engraftment levels were assessed by the determination of the proportion of CD45.1 PB donor cells compared to CD45.2 PB competitor cells 2 months after transplantation.

Data analysis and statistics

Values are presented as the mean, median or cell number ± standard error of the mean (SEM). Statistical comparisons between groups were done using the Student's t-test, $P<0.05$ (*) and $P<0.001$ (**).

Results

A synthetic octosaccharide can mobilize hematopoietic stem cells

New synthetic oligosaccharides, hexa- (EP80006) and octo- (EP80031) saccharides, mimicking the structure of heparan sulfate[26] were designed to compete efficiently with endogenous heparan sulfate. These two oligosaccharides differ in the number of sugar repetitions (Figure 1A) and possess an unnatural substituent group that increases their affinity and/or selectivity (*data not shown*). To characterize the ability of these compounds to mobilize hematopoietic cells, EP80006 and EP80031 (15 mg/kg) were intravenously injected into C57BL/6 mice and peripheral WBC were counted at different times (Figure 1B, left panel). Injection of EP80031 resulted in marked mobilization with three times more WBC than control 30 min to 1 hour after injection, whereas injection of EP80006 resulted in modest WBC mobilization only 30 min after the injection, indicating that EP80031 could be an efficient compound for mobilization.

Compared to phosphate-buffered saline (PBS), injection of EP80031 resulted in a maximum 5-fold increase in the number of peripheral blood c-Kit$^+$Lin$^-$Sca$^+$ (KLS) hematopoietic cells enriched in HSC (Figure 1B, right panel). No differences in the amount of WBC and KLS cells mobilized could be detected with increased concentrations of EP80031 (*data not shown*) and similar results were obtained when a different mouse strain (DBA/2) was used (*data not shown*), indicating that EP80031 was efficient in the two different mouse strains. Taken together these results show that EP80031 should be an efficient compound to mobilize HSPC.

During HSPC mobilization with G-CSF,[34] high levels of blood serum VEGF and SDF-1 could be detected. Thirty minutes after injection of EP80031, the VEGF concentration in serum increased 8-fold compared to that produced by PBS injection (EP80031: 509.6±33 *versus* PBS: 80.7±8 pg/mL) (Figure 1C left panel) and this concentration remained stable for at least 5 h, i.e. when numbers of mobilized WBC or KLS cells declined (Figure 1B, right panel). The serum concentration of SDF-1 paralleled the WBC or KLS cell mobilization perfectly (Figure 1C, right panel). These results indicate that injection of EP80031 was associated with increased serum VEGF and SDF-1 concentrations, similar to those observed during G-CSF-induced HSPC mobilization.[34]

EP80031 has the same efficiency as granulocyte colony-stimulating factor or AMD3100 at mobilizing hematopoietic stem and progenitor cells

As injection of EP80031 resulted in rapid and transient HSPC mobilization and its effects were compared to those of AMD3100, a compound known to mobilize hematopoietic cells and HSC rapidly.[14,35-37] Thirty minutes after injection of EP80031, WBC could be detected in PB whereas AMD3100 resulted in modest WBC mobilization 1 h after injection (Figure 2A, upper panel). In addition, EP80031 mobilized more WBC than AMD3100 (13,300±2,910.3 WBC/μL *versus* 7,700±1,464 WBC/μL 1 h after injection) (Figure 2A, upper panel). EP80031 mobilized more B220$^+$ B-lymphocytes and CD3$^+$ T-lymphocytes and, to a lesser extent, CD11b$^+$ myeloid cells than AMD3100, but injection of both products resulted in the same 6-fold increase in KLS mobilization in the bloodstream 1 h after treatment (Figure 2A, middle and lower panels), a mobilization that is similar to that obtained after G-CSF treatment (*data not shown*).

The clonogenic potential of mobilized PB HSPC was evaluated after injection of G-CSF, AMD3100 or EP80031. Blood cells from mice treated with G-CSF, AMD3100 or EP80031 were cultured in semi-solid methylcellulose and CFU were quantitatively and qualitatively scored after 7 days of culture. The total number of CFU was similar after EP80031, AMD3100 or G-CSF treatment (EP80031: 590±50 *versus* AMD3100: 690±60 and G-CSF: 630±78 CFU/mL) (Figure 2C, left panel). However, the colonies obtained were qualitatively different: CFU-G was the predominant colony type observed after AMD3100 injection (310±22 CFU/mL), CFU-GM was the most abundant colony type found after

G-CSF treatment (243±24 CFU/mL), whereas, after injection of EP80031, the numbers of CFU-G, CFU-GM and CFU-GEMM were similar (180±21, 146±38 and 150±12 CFU/mL, respectively) (Figure 2C, left panel). Finally, G-CSF and EP80031 treatments resulted in higher concentrations of immature myeloid colonies (CFU-GEMM) compared to AMD3100 treatment (G-CSF: 115±15 CFU/mL; EP80031: 151±11 CFU/mL and AMD3100: 105±16 CFU/mL) (Figure 2C, left panel).

Taken together, these results show that EP80031 has the same capacity to mobilize hematopoietic cells enriched in HSC as G-CSF and AMD3100 but with different kinetics. In addition, EP80031 treatment results in increased number of the multi-potential CFU-GEMM compared to the numbers produced by AMD3100 and G-CSF treatments, suggesting a better ability to mobilize immature hematopoietic cells.

EP80031 can act in synergy with the granulocyte colony-stimulating factor + AMD3100 combination to mobilize hematopoietic stem and progenitor cells

To determine whether EP80031 acted on pathways already activated by G-CSF and/or AMD3100 to mediate the mobilization of WBC and HSPC, mobilization mediated by combinations of EP80031 with G-CSF, with AMD3100 or with G-CSF + AMD3100 were compared to mobilization induced by G-CSF + AMD3100. Addition of EP80031 resulted in a higher number of mobilized WBC compared to control and the combination of G-CSF and AMD3100 1 h after AMD3100 or EP80031 injection (G-CSF + EP80031: 25,300 ± 5,131 WBC/μL; AMD3100 + EP80031: 22,700 ± 6,027 WBC/μL; G-CSF + AMD3100 + EP80031: 26,200 ± 1,237 WBC/μL and G-CSF + AMD3100: 17,000 ± 2,430 WBC/μL) (Figure 2B, upper panel). This increased number of WBC in PB was due to an increased number of all mature leukocytes except with the AMD3100 + EP80031 combination that preferentially mobilized B220+ B and CD3+ T-lymphocytes (Figure 2B, middle and lower left panels). Finally, adding EP80031 to the G-CSF + AMD3100 combination resulted in a 3-fold increased number of KLS cells in PB when compared to the others combinations of compounds (Figure 2B, lower right panel). Four days of treatment with G-CSF alone increased mobilization of $B220^+$ lymphocytes, $CD11b^+$ and KLS cells in PB, as previously shown.[21,38] Combining this treatment with AMD3100 and EP80031 resulted in a similar increase of WBC, $B220^+$ lymphocytes, $CD3^+$ T-lymphocytes and KLS cells as that produced by 2 days of treatment with the previously used G-CSF (*Online Supplementary Figure S1A and S1B*). Interestingly, adding EP80031 to the 2 days of treatment with the G-CSF + AMD3100 combination resulted in a similar number of mobilized KLS as that produced by 4 days of treatment with G-CSF + AMD3100 indicating that the use of EP80031 could shorten the G-CSF treatment by 2 days to get the same amount of mobilized KLS cells (*Online Supplementary Figure S1B, right panel*). These results indicate that EP80031 could act in synergy with the G-CSF + AMD3100 combination to mobilize HSPC and might, therefore, be a good candidate to increase the amount of HSC that could be obtained from donors for hematopoietic grafts.

CFU assays were performed on mobilized PB HSPC after

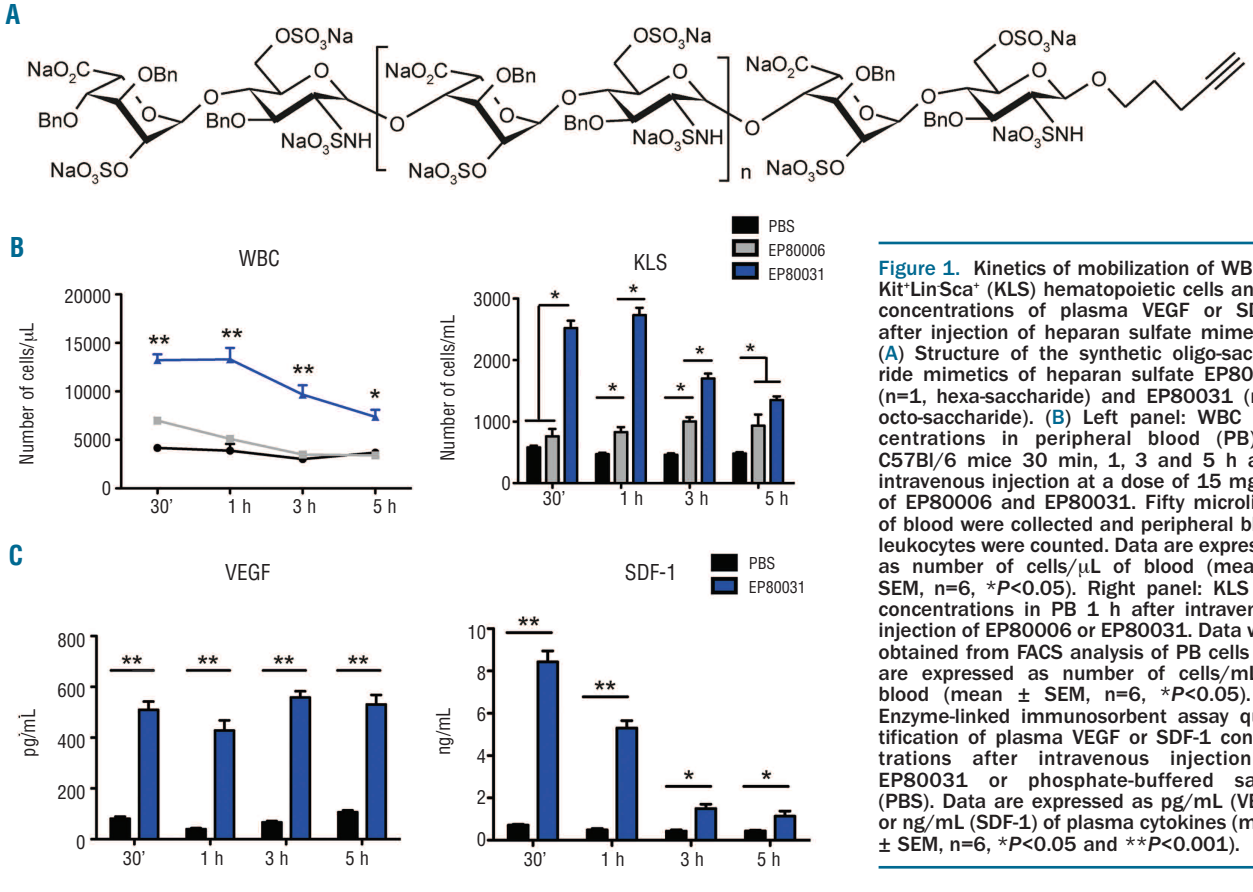

Figure 1. Kinetics of mobilization of WBC, c-$Kit^+Lin^-Sca^+$ (KLS) hematopoietic cells and of concentrations of plasma VEGF or SDF-1 after injection of heparan sulfate mimetics. (**A**) Structure of the synthetic oligo-saccharide mimetics of heparan sulfate EP80006 (n=1, hexa-saccharide) and EP80031 (n=2, octo-saccharide). (**B**) Left panel: WBC concentrations in peripheral blood (PB) of C57Bl/6 mice 30 min, 1, 3 and 5 h after intravenous injection at a dose of 15 mg/kg of EP80006 and EP80031. Fifty microliters of blood were collected and peripheral blood leukocytes were counted. Data are expressed as number of cells/μL of blood (mean ± SEM, n=6, **P*<0.05). Right panel: KLS cell concentrations in PB 1 h after intravenous injection of EP80006 or EP80031. Data were obtained from FACS analysis of PB cells and are expressed as number of cells/mL of blood (mean ± SEM, n=6, **P*<0.05). (**C**) Enzyme-linked immunosorbent assay quantification of plasma VEGF or SDF-1 concentrations after intravenous injection of EP80031 or phosphate-buffered saline (PBS). Data are expressed as pg/mL (VEGF) or ng/mL (SDF-1) of plasma cytokines (mean ± SEM, n=6, **P*<0.05 and ***P*<0.001).

injection of combinations of compounds. Blood cells from mice treated with combinations were cultured in methylcellulose and colonies were quantitatively and qualitatively scored after 7 days of this culture. Three times more colonies were obtained with the triple combination G-CSF + AMD3100 + EP80031 (5,500 ± 600 CFU/mL) when compared to the other combinations (G-CSF + EP80031: 2,060 ± 150 CFU/mL, AMD3100 + EP80031: 1,920 ± 180 CFU/mL and G-CSF + AMD3100: 1,880±200 CFU/mL) (Figure 2C, right panel), and this increase correlated with the increase in KLS cells found in the blood of treated mice (Figure 2B, lower right panel). Even if differences were found in the repartition of the different myeloid lineage-committed colonies between the combinations of compounds, the

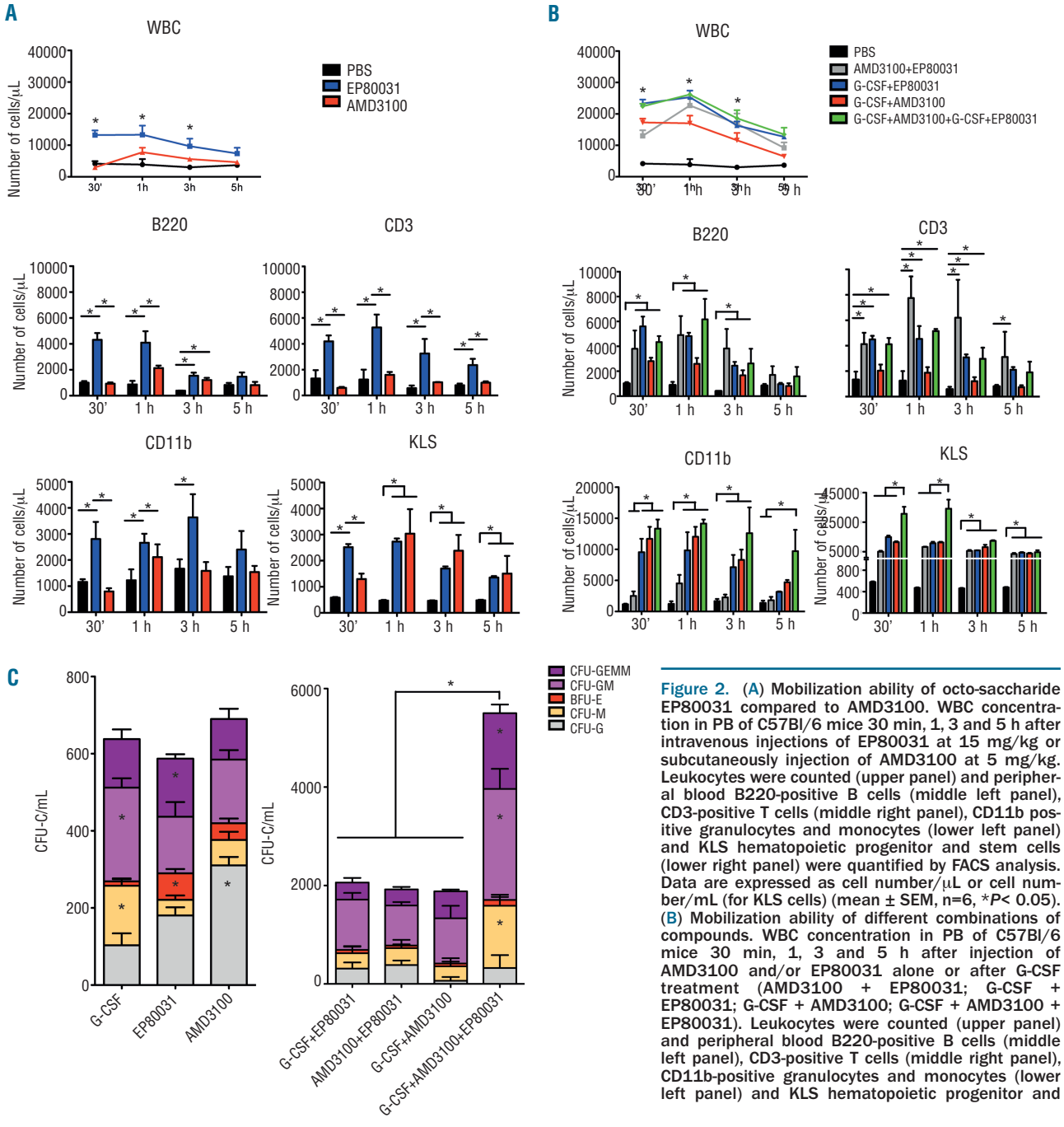

Figure 2. (A) Mobilization ability of octo-saccharide EP80031 compared to AMD3100. WBC concentration in PB of C57Bl/6 mice 30 min, 1, 3 and 5 h after intravenous injections of EP80031 at 15 mg/kg or subcutaneously injection of AMD3100 at 5 mg/kg. Leukocytes were counted (upper panel) and peripheral blood B220-positive B cells (middle left panel), CD3-positive T cells (middle right panel), CD11b positive granulocytes and monocytes (lower left panel) and KLS hematopoietic progenitor and stem cells (lower right panel) were quantified by FACS analysis. Data are expressed as cell number/µL or cell number/mL (for KLS cells) (mean ± SEM, n=6, *P< 0.05). (B) Mobilization ability of different combinations of compounds. WBC concentration in PB of C57Bl/6 mice 30 min, 1, 3 and 5 h after injection of AMD3100 and/or EP80031 alone or after G-CSF treatment (AMD3100 + EP80031; G-CSF + EP80031; G-CSF + AMD3100; G-CSF + AMD3100 + EP80031). Leukocytes were counted (upper panel) and peripheral blood B220-positive B cells (middle left panel), CD3-positive T cells (middle right panel), CD11b-positive granulocytes and monocytes (lower left panel) and KLS hematopoietic progenitor and stem cells (lower right panel) were quantified by FACS analysis. Data are expressed as cell number/µL or cell number/mL (for KLS cells) (mean ± SEM, n=6, *P<0.05). (C) Left panel: clonogenic capacity of EP80031-mobilized hematopoietic cells compared to G-CSF or AMD3100 mobilization. A CFU assay was performed with PB cells 1 h after the injection of EP80031, AMD3100 or 1 h after the last injection of G-CSF. Bar graphs show the number of colony forming unit-granulocytes (CFU-G), colony forming unit-macrophages (CFU-M), erythroid burst-forming units (BFU-E), colony forming unit-granulocytes and macrophages (CFU-GM) and colony forming units with mixed populations of erythroid and myeloid cells (CFU-GEMM) colonies scored after 7 days of culture. Right panel: clonogenic capacity of mobilized hematopoietic cells after injection of different combinations of compounds. Bar graphs show the number of CFU-G, CFU-M, BFU-E, CFU-GM and CFU-GEMM. Data are expressed as CFU-C/mL of blood (mean ± SEM, n=5, *P<0.05).

overall effect resulted in an increase of more immature myeloid colonies (CFU-GM + CFU-GEMM) (Figure 2C, right panel). Four days of treatment with G-CSF alone or in combination with AMD3100 resulted in a 1.5-fold increase in the number of CFU. Adding EP80031 to the 4 days of treatment with the G-CSF + AMD3100 combination resulted in a 2-fold increase in the total number of CFU and CFU-GEMM, and a 3-fold increase in the number of CFU-G and CFU-GM (*Online Supplementary Figure S1C, right panel*). Finally, the total number of CFU after 2 days of treatment with the G-CSF + AMD3100 + EP80031 combination was increased 2-fold when compared to that after 4 days of treatment with the G-CSF + AMD3100 combination (5,500±600 CFU/mL *versus* 2,780±110 CFU/mL, respectively) (*Online Supplementary Figure S1C, middle and right panels*).

These results show that adding EP80031 to the G-CSF + AMD3100 combination greatly increased the number of hematopoietic progenitors without affecting their clonogenic capacity and indicates that EP80031 most probably enhances the effects of G-CSF and/or AMD3100.

Hematopoietic stem cells mobilized by the granulocyte colony-stimulating factor + AMD3100 + EP80031 combination reconstitute long-term hematopoiesis

We studied whether the 3-fold increased number of mobilized KLS had the capacity to reconstitute hematopoiesis using a long-term repopulating assay. Lethally γ-irradiated mice were engrafted with PB KLS cells (15,000 cells) mobilized with G-CSF, G-CSF + AMD3100, G-CSF + EP80031 or G-CSF + AMD3100 + EP80031 from C57Bl/6 CD45.1 donor mice in competition with KLS competitor cells (3,000 cells) isolated from bone marrow of non-conditioned C57Bl/6 CD45.2 mice (Figure 3A). Chimerism was studied 4 months after transplantation in BM of transplanted mice. Animals transplanted with PB KLS cells mobilized with G-CSF + AMD3100 or G-CSF + AMD3100 + EP80031 showed the greatest chimerism with a 3-fold increase in CD45.1 cells when compared to animals grafted with cells mobilized by G-CSF alone (Figure 3B). No statistical difference was observed in CD45.1 chimerism from mice transplanted with KLS mobilized with the G-CSF + AMD3100, G-CSF + EP80031 or G-CSF + AMD3100 + EP80031 combinations (Figure 3B). These results indicate an increase in PB KLS transplantation efficiency when AMD3100, EP80031 or AMD3100 + EP80031 was added to G-CSF to mobilize PB KLS cells.

No difference in total (*data not shown*) or CD45.1+ mature B- and T-lymphocytes, monocytes/granulocytes and KLS cells was found between the G-CSF + AMD3100 or G-CSF + AMD3100 + EP80031 combination in blood (*data not shown*) and bone marrow (Figure 3C).

To study mobilization of long-term hematopoietic stem cells (LT-HSC), secondary transplants were performed. Four months after the secondary transplant, the same CD45.1 chimerism was found as after the primary transplant (Figure 3D, left panel), with similar reconstitutions of the different hematopoietic lineages and progenitor cells (Figure 3D, middle and right panels). These results showed

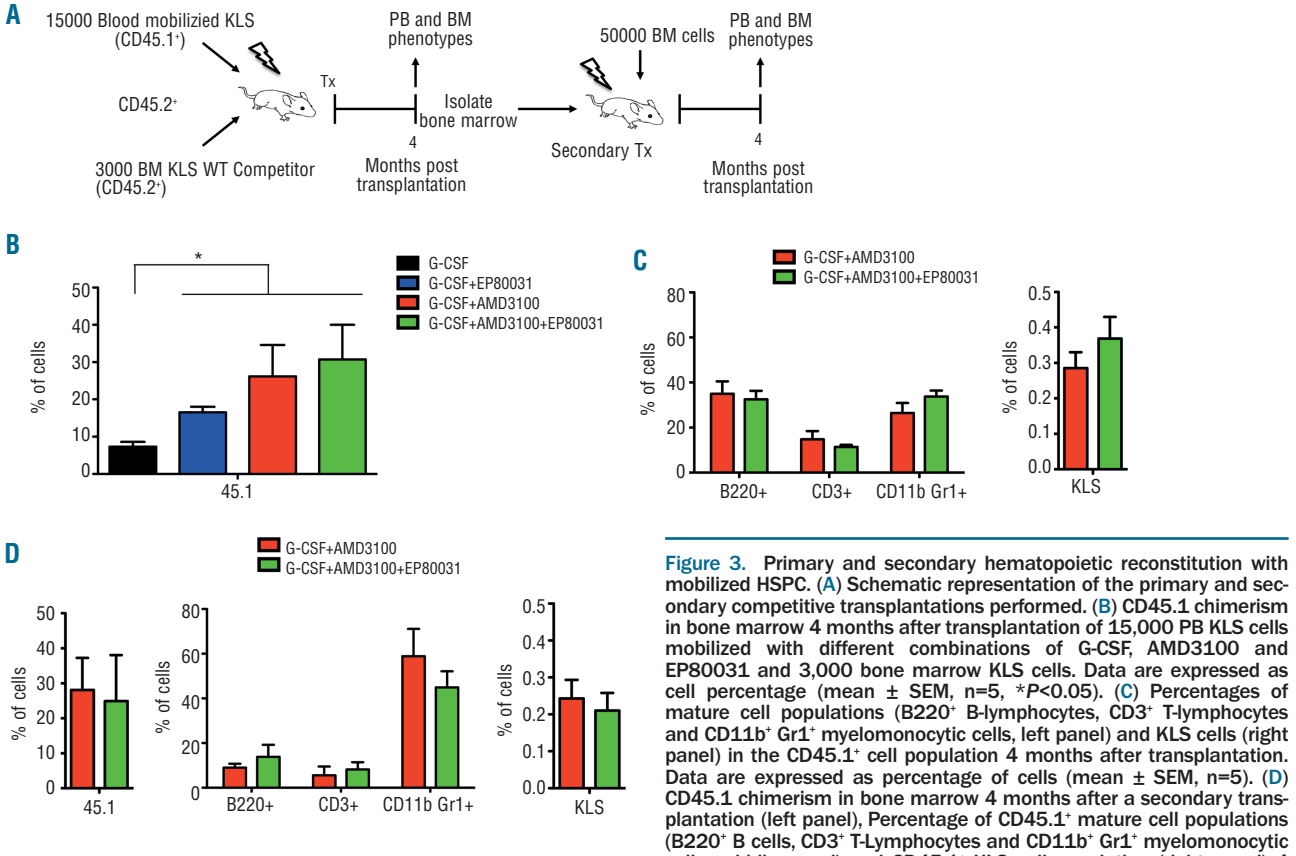

Figure 3. Primary and secondary hematopoietic reconstitution with mobilized HSPC. (A) Schematic representation of the primary and secondary competitive transplantations performed. (B) CD45.1 chimerism in bone marrow 4 months after transplantation of 15,000 PB KLS cells mobilized with different combinations of G-CSF, AMD3100 and EP80031 and 3,000 bone marrow KLS cells. Data are expressed as cell percentage (mean ± SEM, n=5, **P*<0.05). (C) Percentages of mature cell populations (B220+ B-lymphocytes, CD3+ T-lymphocytes and CD11b+ Gr1+ myelomonocytic cells, left panel) and KLS cells (right panel) in the CD45.1+ cell population 4 months after transplantation. Data are expressed as percentage of cells (mean ± SEM, n=5). (D) CD45.1 chimerism in bone marrow 4 months after a secondary transplantation (left panel), Percentage of CD45.1+ mature cell populations (B220+ B cells, CD3+ T-Lymphocytes and CD11b+ Gr1+ myelomonocytic cells, middle panel) and CD45.1+ KLS cell population (right panel) 4 months after secondary transplantation. Data are expressed as percentage of cells (mean ± SEM, n=5).

the capacity of HSC mobilized by the G-CSF + AMD3100 + EP80031 combination to reconstitute long-term hematopoiesis.

To determine whether EP80031 in combination with G-CSF and/or AMD3100 could enhance the engraftment of the mobilized cells above that of G-CSF and/or AMD3100, limiting dilution analysis in a competitive transplant model *in vivo* was performed as previously described.[14] Briefly, dilution of PB leukocytes from CD45.1 mice mobilized with G-CSF, G-CSF + AMD3100 and G-CSF + AMD3100 + EP80031 were transplanted in competition with 5×10^5 BM CD45.2 cells into lethally irradiated mice. Two months after transplantation, the CD45.1 chimerism of mice transplanted with G-CSF mobilized CD45.1$^+$ leukocytes was lower at all ratios used and 50% chimerism was obtained at a 0.5:1 ratio whereas 85% CD45.1 chimerism was obtained in mice transplanted with leukocytes mobilized with G-CSF + AMD3100 or G-CSF + AMD3100 + EP80031 at a 0.5:1 ratio (Figure 4). At lower ratios i.e. 0.25:1 and 0.1:1, CD45.1 chimerism of mice transplanted with G-CSF + AMD3100 + EP80031 mobilized leukocytes remained significantly higher than for the ones transplanted with G-CSF + AMD3100 (Figure 4). Finally, when a 0.05:1 ratio was used, a 5-fold higher CD45.1 chimerism was obtained in mice transplanted with G-CSF + AMD3100 + EP80031 mobilized leukocytes when compared to G-CSF + AMD3100 mobilized leukocytes (35±2.5% triple combination *versus* 7±2.2% double combination) (Figure 4).

All these results highlight the ability of EP80031 in combination with G-CSF + AMD3100 to enrich the graft in hematopoietic cells that increased hematopoietic reconstitution.

Discussion

Recruitment of hematopoietic cells from the BM occurs following various stressful situations such as inflammation, bleeding, chemotherapy, or administration of cytotoxic drugs.[21] This course is also used to actively separate HSC from their BM niches and let them join the bloodstream to constitute a graft containing a high number of mobilized HSC. The retention and egress of HSC in and from the BM are regulated by the ECM; heparan sulfate proteoglycans are one of major components of the ECM.[26] Heparan sulfate proteoglycans are composed of multiple forms of heparan sulfate and play a key role in the interaction between hematopoietic stem and/or progenitor cells and humoral components of the BM ECM.[30,39] Exogeneous heparan sulfate could mobilize HSC, probably through titration of heparanases, endonucleases that cleave heparan sulfates,[32,40] as heparanase down-regulation is associated with HSC expansion and mobilization.[41,42]

In this study, we analyzed the effects of a new class of oligosaccharides, heparan sulfate mimetics, on HSC mobilization in peripheral blood. These oligosaccharides can efficiently compete with endogenous heparan sulfates and possess a variety of structures that can be used to strengthen their affinity and/or their selectivity towards target proteins. To characterize the ability of these compounds to mobilize HSC, the two oligosaccharides EP80006 and EP80031 were intravenously injected into mice and the animals' PB cells were then analyzed. EP80031 showed a high capacity to mobilize WBC and KLS cells in the bloodstream while EP80006 showed only a modest mobilizing effect. This different ability in mobilizing cells could be due to the difference in the number of sugar repeats in the oligosaccharides as it was demonstrated that the functions and capacity to bind proteins of stromal-derived heparan sulfate is related to the length of the polysaccharide chain and its pattern of sulfation.[43-45]

After EP80031 treatment, the number of KLS cells in PB increased rapidly and the kinetics of KLS mobilization correlated with increases of serum SDF-1 and VEGF concentrations. These finding support: (i) the link between plasma SDF-1 levels and HSPC egress from the bone marrow, a capacity associated with activation of the metalloproteinase MMP-9;[46,47] and (ii) involvement of the VEGF pathway in mobilization and recruitment of HSC.[48] It was also recently shown that rapid mobilization of HSPC by AMD3100 might be accounted for by a CXCR4-dependent SDF-1 release into the circulation of mice.[47] When compared to AMD3100, EP80031 had greater potential to increase PB leukocyte concentration and a similar ability to mobilize HSC. The difference in the kinetics of WBC mobilization by AMD3100 and EP80031 might be explained, in part, by the different route of administration (intravenous for EP80031 and subcutaneous for AMD3100) but, more likely, by the activation of different retention/egress pathways. EP80031 as AMD3100 might act on the CXCR4/SDF-1 axis to mobilize hematopoietic cells as we observed an increased plasma level of SDF-1 after EP80031 injection. However, heparan sulfates also regulate the sphingosine 1-phosphate (S1P) signaling pathway[49] which controls recirculation of lymphocytes[50] and egress of HSPC from the BM,[51] suggesting that EP80031 might also act on this pathway to enhance hematopoietic cell mobilization.

A synergic and potentiating effect on KLS cell mobilization was shown when EP80031 was added to G-CSF or/and AMD3100 treatment. As the number of WBC in PB after G-CSF + AMD3100 + EP80031 treatment was only increased

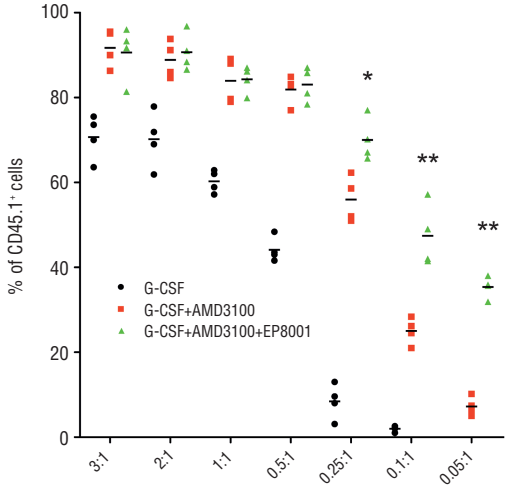

Figure 4. CD45.1 chimerism in peripheral blood (PB) of mice transplanted with PB cells mobilized with G-CSF, G-CSF + AMD3100 or G-CSF + AMD3100 + EP80031 and competitive BM cells 2 months after transplantation. The ratio of donor (CD45.1) blood cells to competitor (CD45.2) BM cells is defined by the number of mononuclear blood cells of X donor mice to a constant number ($5x10^5$) competitor BM cells. Data are expressed as cell percentage (mean ± SEM, n=4, *P<0.05).

1.5 times compared to that after G-CSF + AMD3100 treatment whereas the number of KLS cells increased more than 3 times, HSC mobilization cannot be explained by the massive egress of hematopoietic cells from the BM after EP80031 treatment but could be due to specific effects on HSC retention. Among the possible EP80031-mediated interferences between HSC and the BM microenvironment, competition with GAG present on VLA-4 or β1 integrin proteins expressed by stromal cells and involved in HSC retention[10] and/or activation of matrix proteases, such as MMP-9,[46] which mediate release of HSC into the bloodstream are good candidates.

Mobilization mediated by EP80031 did not interfere with the clonogenicity of the mobilized HSPC and EP80031 or G-CSF treatment resulted in increased mobilization of immature hematopoietic progenitors indicating an important role of the ECM and its heparan sulfate components in the retention of HSPC in BM. In addition, EP80031 not only preserved the quality of the mobilized HSPC but also increased their number when used in combination with G-CSF + AMD3100 and more when G-CSF treatment was given for 4 days. This increase was also found on HSC as adding EP80031 to the G-CSF + AMD3100 combination resulted in better grafts even in secondary transplants. Moreover, treatment with G-CSF + AMD3100 + EP80031 greatly enhanced the potential of mobilized blood leukocytes to reconstitute hematopoiesis after competitive transplantation. This suggested that addition of EP80031 might: (i) increase the number and/or quality of mobilized HSC and/or (ii) mobilize hematopoietic cells that facilitate engraftment as recently described with AMD3100, which increases the number of mobilized regulatory T cells.[52]

Although previous studies using sulfated glycans have demonstrated mobilization of HSPC and short-term HSC,[40,53] this study shows, for the first time, that heparan sulfate mimetics can be used to greatly increase mobilization of long-term HSC. EP80031 defines a new family of efficient mobilizing agents as it could alone, or in combination with G-CSF and/or AMD3100, mobilize a high number of HSC without interfering with their quality and functionality. The use of mimetics of small-glyco drugs based on heparan sulfate can, therefore, be foreseen to improve stem cell mobilization, particularly in the prospect of therapeutic use in humans.

Authorship and Disclosures

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