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Viagra Enables Efficient, Single-Day Hematopoietic Stem Cell Mobilization

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SUMMARY

Hematopoietic stem cell (HSC) transplantation is a curative treatment for a variety of blood and immune disorders. Currently available methods to obtain donor HSCs are suboptimal, and the limited supply of donor HSCs hampers the success and availability of HSC transplantation therapies. We recently showed that manipulation of vascular integrity can be employed to induce HSC mobilization from the bone marrow to the blood stream, facilitating non-invasive collection of HSCs. Here, we tested whether FDA-approved vasodilators are capable of mobilizing HSCs. We found that a rapid, 2-h regimen of a single oral dose of Viagra (sildenafil citrate) combined with a single injection of the CXCR4 antagonist AMD3100 leads to efficient HSC mobilization at levels rivaling the standard-of-care 5-day regimen of granulocyte-colony stimulating factor (G-CSF/Filgrastim/Neupogen). Our findings solidify vascular integrity as an essential regulator of HSC trafficking and provide an attractive, single-day regimen for HSC mobilization using already FDA-approved drugs.

INTRODUCTION

Hematopoietic stem cells (HSCs) have the capacity for lifelong cures of a variety of disorders, but significant hurdles have caused hematopoietic cell therapies (HCTs) to be reserved mainly for patients with malignant disease that have run out of other treatment options. One such hurdle is a limited supply of donor HSCs for transplantation and efficient methods to obtain them (Giralt et al., 2014; Körbling and Freireich, 2011). Originally, HSC-containing samples were harvested by extraction from the bone marrow (BM), but development of efficient and well-tolerated strategies for mobilizing HSCs to the blood stream has established mobilized blood as the most common source of cells for hematopoietic transplantation. Although less invasive than BM extraction, the current standard of harvesting hematopoietic stem and progenitor cells (HSPCs) from the blood of donors treated with granulocyte-colony stimulating factor (G-CSF) for several days is complex, costly, unsuccessful in a significant proportion of donors due to previous chemotherapy, population variability, and unknown factors, and too frequently results in morbidities, such as fatigue, nausea, and bone pain (Murata et al., 1999; Navarro et al., 2013; To et al., 2011). The CXCR4 antagonist AMD3100 rapidly and reproducibly mobilizes HSCs (Broxmeyer et al., 2005; Couban et al., 2019; Devine et al., 2008), but, because of its relative inefficiency as a single agent HSC mobilizer, it is more commonly used as a salvaging agent when the standard G-CSF regimen fails (Douglas et al., 2018; Giralt et al., 2014). Even though G-CSF-mediated mobilization is successful in most donors, improved harvesting protocols would significantly improve the success rate for current indications and open curative HCT to a wider spectrum of disorders. New mobilization regimens can also reveal mechanisms of HSC retention in the BM. Despite the use of HSCs in clinical therapy for over half a century and the urgent need for improved strategies, the mechanisms regulating HSC trafficking remain unclear. We recently reported that the transmembrane receptor ROBO4 regulates directional trafficking of HSCs across the vascular endothelium to and from the BM (Smith-Berdan et al., 2011, 2015). Deletion of ROBO4 results in increased vascular permeability (Jones et al., 2008; Smith-Berdan et al., 2015) and increased numbers of HSCs in the blood stream (Smith-Berdan et al., 2015). Upon testing the effects of induced vascular permeability on HSC trafficking, we reported increased vascular leakage as well as 2- to 3-fold increases in HSC numbers in the blood within 15 min of an intravenous administration of $rhVEGF_{164}$ or histamine (Smith-Berdan et al., 2015). This revealed that induced vascular permeability, by itself, is sufficient to mobilize HSCs. In addition, we showed that VEGF-induced vascular permeability significantly improved AMD3100-mediated mobilization of engraftable HSCs (Smith-Berdan et al., 2015). Subsequently, Pelus and colleagues reported similar findings using neuropeptide Y (Singh et al., 2017) and GROβ, a CXCR2 agonist that induces vascular permeability (Hoggatt et al., 2018). Because pharmacokinetics and allelic variations in CXCR2 preclude effective or universal use of VEGF (Giacca and Zacchigna, 2012) and GROβ (Hoggatt et al., 2018) in humans, we investigated whether currently FDA-approved vasomodulators can be repurposed for HSC mobilization. Here, we tested the effects of Viagra (sildenafil citrate), a phosphodiesterase type 5 (PDE5) inhibitor, on HSC mobilization. PDE5 inhibitors block degradation of cyclic GMP in the smooth muscle cells lining blood vessels, resulting in vasodilation. This inhibition is immediate, with a peak activity 2 h after oral administration of the drug (Andersson, 2018). Viagra was originally developed to combat high

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blood pressure, coronary heart disease, and angina pectoris, and is currently successfully used to treat a variety of vascular disorders, including pulmonary hypertension, altitude sickness, Raynaud's syndrome, preeclampsia, and erectile dysfunction (Andersson, 2018). Here, we reveal that Viagra should also be considered in combination therapy for very rapid and efficient, single-day HSC mobilization.

RESULTS

To test whether sildenafil citrate (Viagra) such as in injections of histamine or VEGF (Smith-Berdan et al., 2015), was capable of mobilizing HSCs, we first administered a single dose of Viagra via oral gavage at 3 mg/kg (Motta et al., 2015; US FDA, 2018) (Figure 1A). Analysis of total perfused blood 2 h after Viagra treatment did not lead to detectable increases in HSCs (defined as cKIT$^+$lineage$^-$SCA1$^+$CD27$^+$FLK2$^-$ cells; Figures 1B and 1C) or other hematopoietic cells (Figure S1A). Similarly, higher doses of Viagra (10 mg/kg) administered as in Figure 1A or for 3 consecutive days failed to mobilize significant numbers of HSCs (Figures S1B–S1D). In contrast, a single subcutaneous dose of the CXCR4 antagonist, AMD3100, a known inducer of HSC mobilization (Broxmeyer et al., 2005), led to modest, but reproducible, HSC mobilization (Figures 1B, 1C, and S1A). We next tested the effects of Viagra combined with AMD3100. Remarkably, a single oral dose of Viagra significantly improved AMD3100-induced HSC mobilization in a rapid, 2-h protocol (Figures 1A–1C). The mobilization was transient, because the numbers of HSCs in the blood had returned to normal 4 h post-treatment (Figure S1E). The HSC mobilization efficiency with 2-h Viagra plus AMD3100 was comparable with that of a 5-day G-CSF protocol (250 μg/kg daily subcutaneous injections) (Figures 1A–1C). Higher doses of Viagra (10 and 30 mg/kg) also improved AMD3100-mediated HSC mobilization, but were not more effective than 3 mg/kg (Figure S1B). Furthermore, a 3-day oral Viagra regimen combined with a single AMD3100 injection led to significantly more HSCs in the bloodstream than AMD3100 alone (Figure S1C and S1D). Compared with control mice, the numbers of phenotypic HSCs increased 2.9-, 7.5-, and 8.4-fold with AMD3100 alone, AMD3100 plus a single Viagra dose, and AMD3100 plus 3 days of Viagra, respectively. The numbers of HSCs in the bloodstream in the rapid (~2,500 HSCs/mouse) and 3-day (~2,800 HSCs/mouse) Viagra/AMD3100 combination were similar to the numbers present 1 day after 4 consecutive days of G-CSF injections (~3,400 HSCs/mouse; Figure 1B). Assessment of vascular permeability by Miles assay revealed that Viagra plus AMD3100 led to increased vascular leak (Figure 1D), consistent with Viagra acting as a vasodilator, and with vascular permeability leading to HSC mobilization (Smith-Berdan et al., 2015; Singh et al., 2017; Hoggatt et al., 2018). Transplantation into lethally (Figures 1E–1G) and sublethally (Figures S1F and S1G) conditioned recipients demonstrated that the blood from mice treated with Viagra plus AMD3100 contained an increased number of functional, long-term engrafting multipotent HSCs compared with the blood from mice treated with AMD3100 alone. Although G-CSF did not lead to significantly higher numbers of phenotypic HSCs in the blood compared with Viagra plus AMD (Figure 1B), blood from G-CSF-mobilized donors reconstituted recipients more robustly (Figures S1H–S1I), consistent with G-CSF selectively mobilizing the most quiescent HSCs (Bernitz et al., 2017). Similarly, the BM of recipients of blood from donors treated with both Viagra and AMD3100 contained significantly more donor HSCs than the BM of recipients of blood cells from mice treated with AMD3100 alone (Figures 1H and S1J). Transplantation of BM cells from lethally irradiated primary recipients into secondary hosts also led to long-term, multilineage engraftment, with Viagra plus AMD3100 outperforming AMD3100 alone (Figure 1I). Thus, the combination of Viagra and AMD3100 enables rapid and efficient mobilization of serially long-term reconstituting, self-renewing, and multipotent HSCs.

DISCUSSION

The value of alternative HSC mobilization regimens to complement G-CSF-based protocols is multiple. G-CSF, and G-CSF plus AMD3100 (Figures S1K and S1L), effectively mobilizes HSCs in most donors and will likely persist as a common clinical procedure. Importantly, however, a mobilization regimen that does not involve cell expansion, yet offers robust yields of HSCs, would increase the availability of HCT for autologous candidates who cannot tolerate the standard G-CSF mobilization treatment, such as the very ill, elderly, or sickle-cell patients (Fitzhugh et al., 2009; Giralt et al., 2014). New mobilization strategies are also needed to reduce the incidence of mobilization failure, which continues to hamper HCT success, especially for patients with a history of chemotherapy (Demirer et al., 1996; Gertz, 2010; Morgan et al., 2004; To et al., 2011). Viagra and other rapidly acting HSC mobilizers such as VEGFR agonists, Neuropeptide Y, the CXCR2 agonist GROβ and the integrin inhibitors Bio5192a and BOP (Cao et al., 2016; Hoggatt et al., 2018; Ramirez et al., 2009; Singh et al., 2017; Smith-Berdan et al., 2015) are promising candidates for overcoming these challenges. Novel regimens may also reduce side effects, including

Figure 1. Viagra Combined with AMD3100 Rapidly and Efficiently Mobilizes Functional HSCs

(A) Experimental design for 2-h HSC mobilization with Viagra plus AMD3100 (top), or 5-day G-CSF treatment (bottom). G-CSF was administered to mice once daily for 4 days (250 μg/kg). Viagra was administered via oral gavage (OG) (3 mg/kg) once, 1 h before a single

(legend continued on next page)

the bone pain and nausea that frequently accompanies G-CSF treatment (Murata et al., 1999; Navarro et al., 2013; To et al., 2011). Importantly, Viagra alone did not lead to detectable increases in HSPCs in the blood, eliminating concerns of inadvertent HSC mobilization in patients using Viagra for other indications. Cost reduction is another consideration. Viagra generics, at ~$2 per human dose equivalent to 3 mg/kg, is cheaper than G-CSF/Neupogen/Filgrastim, which costs ~$3,800 per treatment regimen (B.C. Cancer, 2018; Consumer Reports, 2017; Shaughnessy et al., 2011; James et al., 2017). Additional cost reductions are also afforded by the simplicity of oral intake of Viagra versus the multi-day injections required for G-CSF. The combined benefits of reduced time commitment and complexity of administration, and the more beneficial side effect profile of Viagra versus G-CSF, would likely attract more volunteer donors and make HCT a reality for additional patient cohorts. Finally, this study solidifies vascular integrity as an essential regulator of HSC trafficking (Hoggatt et al., 2018; Singh et al., 2017; Smith-Berdan et al., 2015). The discovery of vasomodulating drugs as HSC mobilizers provides new mechanistic insights into the regulation of HSC location and inspires additional investigations to control cellular trafficking.

EXPERIMENTAL PROCEDURES

Mouse Lines

All animals were housed and bred in the AALAC-accredited vivarium at the University of California Santa Cruz (UCSC). C57BL6 (JAX cat. no. 000664), B6 CD45.1 BoyJ (JAX cat. no. 002014), and UBC-GFP (JAX cat. no. 004353) mice were maintained according to approved protocols by UCSC's Institutional Animal Care and Use Committee. Adult mice were used between 8 and 16 weeks of age and randomized based on sex.

Mobilization/Tissue Isolation

Mice were treated with subcutaneous injections of rhG-CSF (Amgen, Thousand Oaks, CA, 250 μg/kg) or a single dose of AMD3100 (Sigma, 2.5 or 5 mg/kg where specified), or by oral gavage with sildenafil citrate (Sigma; 3, 10, or 30 mg/kg) (Motta et al., 2015) as indicated. Maximum blood was obtained by perfusing the mouse with PBS/20 mM EDTA through the left ventricle, clipping the right atrium and collecting the pooled perfused blood in the chest cavity. The total blood was processed for cell counts and flow cytometry analysis, as described previously (Smith-Berdan et al., 2011, 2015). In brief, cells were pelleted by centrifugation, washed with 2% donor calf serum in PBS to remove EDTA, then incubated with fluorescently conjugated antibodies for flow cytometry analysis or resuspended in HBSS for transplantation into conditioned recipients.

Transplantation of Mobilized HSPCs

Reconstitution assays were performed by transplanting half of the total cells obtained by perfusion per mouse into sublethally irradiated hosts (742 rads) and a third of the total cells obtained by perfusion per mouse into lethally irradiated hosts (1,025 rads). Donor mice were either C57BL6 or UBC-GFP, transplanted into congenic B6 CD45.1 BoyJ or C57BL6 irradiated hosts (Boyer et al., 2019; Smith-Berdan et al., 2015). Recipient mice were bled at the indicated intervals post-transplantation via the tail

subcutaneous (SQ) injection of AMD3100 (2.5 mg/kg). Blood was collected by perfusion 1 h after AMD3100 or 24 h after G-CSF and analyzed by flow cytometry (C) and multilineage reconstitution of lethally irradiated recipients (E–G).

(B) One oral dose of Viagra significantly improves AMD3100-mediated HSC mobilization compared with AMD3100 alone. Five-day, multi-dose G-CSF mobilization was not significantly better than the 2-h Viagra + AMD3100 mobilization protocol. N = 7–27 mice per cohort in 5 independent experiments. One-way ANOVA; $p = 0.0005$. Tukey multi-parameter test; NS, not significant; **$p < 0.001$, ***$p < 0.0005$, ****$p < 0.0001$.

(C) Representative flow cytometry plots of mobilized blood from (B).

(D) Vascular permeability increases after Viagra plus AMD3100 treatment in both the bone marrow and small intestine. Treatment schedule as in (A), with vascular permeability tested 1 h post-AMD3100 injection by Miles assay. Data represent three independent experiments, N = 6–12 mice per cohort. One-way ANOVA; $p = 0.0036$. Tukey multi-parameter test; *$p < 0.05$, **$p < 0.001$, ****$p < 0.0001$.

(E) Donor chimerism over 5 months in lethally irradiated mice transplanted with blood from mice mobilized with AMD3100 alone or with both Viagra and AMD3100 as in the schedule shown in (A). UBC-GFP mice were used as the mobilized donor mice, enabling identification of donor-derived (GFP+) cells versus the unlabeled cells of the wild-type recipients. N = 5–7 mice per cohort in 3 independent experiments. Paired t test; *$p < 0.05$ and **$p < 0.01$.

(F) Representative flow cytometry plots of UBC-GFP donor chimerism in the peripheral blood for B, T, and GM cells, platelets, and erythrocytes 20 weeks post-transplantation into lethally irradiated recipients from (E).

(G) Quantification of leukocyte lineage distribution from donor-derived cells 20 weeks post-transplantation in the mice from (E) and (F).

(H) Blood from Viagra + AMD3100 mobilized mice reconstitute HSCs in the bone marrow of recipient mice. Paired t test; *$p < 0.05$.

(I) Long-term multilineage engraftment upon serial transplantation of bone marrow cells from the primary recipients in (E–H) into secondary, lethally irradiated wild-type hosts. Total donor chimerism in the peripheral blood over the course of the experiment is shown on the left. Quantification of donor-derived B, T, and GM cells, platelets, and erythrocytes 20 weeks post-transplantation is shown on the right. Data represent three independent experiments, N = 9–12 mice per cohort. Unpaired t test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
See also Figure S1.

vein for peripheral blood analysis, and BM cells were harvested >16 weeks post-transplantation. For original data, please contact cforsber@ucsc.edu.

Flow Cytometry

Cell labeling was performed on ice in 1× PBS with 5 mM EDTA and 2% serum. Antibodies used are listed in the Supplemental Information. Samples were analyzed for donor chimerism (detectable by either GFP or antibodies targeted at the CD45.1/2 locus) on LSRII or AriaIII equipment (Becton Dickinson, San Jose, CA), as described previously (Beaudin et al., 2016; Boyer et al., 2019, 2011; Leung et al., 2019; Smith-Berdan et al., 2015, 2011; Ugarte et al., 2015).

Vascular Permeability

A modified Miles assay was utilized to assess *in vivo* vascular permeability (Miles and Miles, 1952; Smith-Berdan et al., 2015). Post-treatment with AMD3100 and/or Viagra, mice were injected IV with Evans Blue (50 mg/kg). Dye was allowed to leak into tissues for 10 min before euthanization by isoflurane inhalation. Vascular leak was measured as OD_{650}/tissue mass after Evans blue extraction from tissues by incubation in formamide for 3–5 h at 55°C.

Quantification and Statistical Analysis

The number of experiments, N, and what N represents can be found in the legend for each figure. Statistical significance was determined by two-tailed unpaired Student's test or one-way ANOVA followed by Tukey multiple comparisons test. All data are shown as mean ± SEM representing at least three independent experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2019.09.004.

AUTHOR CONTRIBUTIONS

S.S.-B. and E.C.F conceived of the project, designed experiments, analyzed data, and co-wrote the manuscript. S.S.-B., A.B., and S.R. performed the experiments.

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