HEMATOPOIETIC STEM CELL SOFTENING MEDIATES MOBILIZATION DUE TO AMD3100, THEREBY INCREASING COUNT IN PERIPHERAL BLOOD

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LIST OF SYMBOLS AND ABBREVIATIONS

HSCs	Hematopoietic Stem Cells
WBCs	White Blood Cells
RBCs	Red Blood Cells
G-CSF	Granulocyte colony-stimulating factor
AMD3100	Plerixafor

SUMMARY

Hematopoietic stem cells (HSCs) have the ability to differentiate into any blood cell as well as self-renew, giving rise to their pluripotent attribute. With the ability to differentiate, HSCs have the potential to be transplanted from healthy donors to matched patients with hematological malignancies as well as bone marrow failure. While the bulk of HSCs are located within the bone marrow, mobilization into the peripheral blood is required for accessible collection of HSCs, which ultimately eliminates the need for surgical procedures. Previous research findings have found that hematopoietic growth factor cytokines, more specifically Granulocyte colony-stimulating factor (G-CSF), as well as the mobilizing agent, plerixafor (AMD3100) increase mobilization of HSCs into the peripheral blood. While G-CSF and AMD3100 have both been scientifically proven and approved to increase HSC mobilization, the mechanical properties of HSCs have yet to be observed when mobilizing from the bone marrow to the peripheral blood. Here we use HSCs flowing through a microfluidic model to represent mobilization and hope to see cell softening due to AMD3100 during transit through the microfluidic device. By investigating the mechanical properties of HSCs during mobilization in the presence of AMD3100, clinical significance can lead to further studies as well as alternative mobilization techniques for use with HSC transplantation for patients with hematological malignancies as well as bone marrow failure.

CHAPTER 1

INTRODUCTION

Hematopoietic stem cells (HSCs) have the ability to differentiate into any blood cell as well as self-renew, giving rise to their pluripotent attribute. HSCs are able to differentiate into white blood cells (WBCs) or leukocytes, lymphoid cells, red blood cells (RBCs) or erythrocytes, and platelets. In order to form the aforementioned blood cells, HSCs must undergo a process termed hematopoiesis within the bone marrow where specific progenitor cells dictate the pathway which the HSCs will take to becoming a specific blood cell (Kohn, Nolta, & Crooks, 2001).

With the ability to differentiate, HSCs have the potential to be transplanted from healthy donors to matched patients with hematological malignancies as well as bone marrow failure. While the bulk of HSCs are located within the bone marrow, mobilization into the peripheral blood is required for accessible collection of HSCs, which ultimately eliminates the need for surgical procedures. Under normal conditions, HSCs within the peripheral blood increase during stressful conditions such as inflammation or bleeding. Previous research findings by Motabi and DiPersio have found that hematopoietic growth factor cytokines, more specifically Granulocyte colony-stimulating factor (G-CSF), as well as the mobilizing agent, plerixafor (AMD3100) increase mobilization of HSCs into the peripheral blood. G-CSF is an approved cytokine that alters the HSC niche, or microenvironment, within the bone marrow and activates proliferation preceding mobilization (Motabi & DiPersio, 2012). On the other hand, AMD3100 is a bicyclam molecule that acts as a reversible antagonist of CXCR4, a receptor for stromal derived factor-1 (SDF-1), which ultimately inhibits SDF-1 to CXCR4 binding and leads to SDF-1 down regulation (Devine et al., 2008). Recently, clinical WBC count has been shown by Fay et al. at the Lam Lab at the Georgia Institute

of Technology to increase via softening due to glucocorticoid and catecholamine hormones which aids in demargination and trafficking. Since, WBCs ultimately stem from HSCs, the research findings provide significance to the mechanical properties of HSCs during mobilization from the bone marrow to the circulating peripheral blood (Fay et al., 2016).

While G-CSF and AMD3100 have both been scientifically proven and approved to increase HSC mobilization, the mechanical properties of HSCs have yet to be observed when mobilizing from the bone marrow to the peripheral blood. Following recent findings relating to leukocytes and questions related to whether changes in mechanical properties affect mobilization, HSC stiffness is explored during mobilization when exposed to G-CSF and AMD3100. A reason for not researching HSC mechanical properties during mobilization, as of yet, may stem from the fact that certain mobilizing agents are known to increase HSCs in the peripheral blood and therefore, knowing how HSCs themselves are affected during mobilization may not be of great importance. But, this gap in understanding may prevent discovery of alternatives to HSC mobilization and ultimately HSC transplantation.

By investigating the mechanical properties of HSCs during mobilization in the presence of G-CSF as well as AMD3100, clinical significance can lead to further studies as well as alternative mobilization techniques for use with HSC transplantation for patients with hematological malignancies as well as bone marrow failure. In addition, a purely mechanical result in increased mobilization will be observed as opposed to the currently accepted mechanisms of G-CSF and AMD3100, respectively. Ultimately, a mechanical understanding of HSCs will allow for a greater understanding of hematopoiesis and mobilization.

CHAPTER 2

LITERATURE REVIEW

Background on Hematopoietic Stem Cells

Before focusing on the process of HSC mobilization, self-renewal, differentiation, and proliferation of HSCs should be understood to develop a background understanding of the potential of HSCs as a form of treatment. Ogawa's results from *in vitro* experiments confirm previous findings that self-renewal and differentiation can be represented as stochastic models, while cell survival and proliferation do not reflect a stochastic model, but are governed by various cytokines that can both stimulate and inhibit the process (Ogawa, 1993). Given that Ogawa's paper is a review, detailed methods, results, and analysis techniques were not stated; only the overall conclusion from the experiments was described. Further experimentation down the line will be necessary to account for *in vivo* affects and technological advancements. Ogawa goes into detail about the mechanism and function of the cytokine, G-CSF, a factor that this research will be using in the experiments, allowing this study to understand the respective interactions at a cellular level when G-CSF is administered. While G-CSF interactions with HSCs are clearly understood, additional information regarding their mechanical properties in the presence of the cytokine is not. This gap in research is the focus of the current study.

Effects of G-CSF and AMD3100

G-CSF, a cytokine, and AMD3100, a mobilizing agent, have been known to increase mobilization of HSCs into peripheral blood from previous studies, while also

being approved as a treatment method. Motabi and DiPersio present an overview of HSC mobilization and the results that AMD3100 affects adhesion and chemical gradients and therefore mobilizes HSCs within hours, while G-CSF affects the microenvironment and therefore requires days before mobilization of HSCs is observed (Motabi & DiPersio, 2012). As reported by Motabi and DiPersio, the results stem from review of prior research findings without performing their own experiments, leading to the ability for future researchers to conduct experiments and confirm findings, similar to Ogawa. By understanding the effects of mobilization due to G-CSF and AMD3100, this research will be able to hypothesize that a change in hematopoietic stem cell mechanical properties will occur when exposed to the respective factor. In addition, the review mentions multiple dosages and time scales patients were exposed to which allows for this research to establish a range of parameters that will be used to treat the hematopoietic stem cells for this experiment. According to Azab et al., AMD3100 is shown to increase apoptosis when bone marrow stromal cells are present, to inhibit adhesion, and to limit CXCR4 expression temporarily before returning to steady state. From in vivo studies, AMD3100 was shown to mobilize myeloma cells and have increased sensitivity and to aid in the reduction of tumors (Azab et al., 2009). While the paper focuses on multiple myeloma cells more than HSCs, AMD3100 and other drugs were used on HSCs in a small portion of the paper. Seeing that HSC mobilization has minimally been tested, this creates a gap in research that will be potentially covered by the findings from the current research. Devine et al. conducted a pilot trial for hematopoietic stem and progenitor cells mobilization via AMD3100 only to inhibit CXCR4 interactions. From the trial, Devine et al. holds the view that AMD3100 increased mean white blood cell count about 3-fold,

while the median CD34 count increased 8-fold (Devine et al., 2008). From Devine et al.'s results, it may follow that mechanical properties of HSC can be studied to determine if a change exists when traversing from the bone marrow to the peripheral blood, giving rise to the research goals.

Mechanical Properties of White Blood Cells

Previous research from the Lam Lab at the Georgia Institute of Technology and Emory University has revealed that glucocorticoids and catecholamine hormones increase clinical white blood cell count via softening due to demargination and trafficking. As Fay et al. states, glucocorticoids and catecholamines cause leukocyte demargination in vessels due mostly to mechanical properties using ex vivo, in vitro, and computational experiments. Through demargination and softening, an increase in white blood cell count is observed in the complete blood count. Using two different microfluidic models, transit times are observed and compared to determine that a faster time suggests deformation while the positioning of the leukocyte in the "vessel" shows demargination, respectively (Fay et al., 2016). The research findings provide significance to the mechanical properties of HSCs during mobilization from the bone marrow to the circulating peripheral blood due to the fact that WBCs ultimately stem from HSCs. While this paper focuses on leukocytes, the same type of experiment will be performed on hematopoietic stem cells since this study will be an extension of this paper. Fay et al. provides a very detailed paper consisting of various methods to affirm the findings of the research, which the research will be following to an extent with the addition of AMD3100 and G-CSF as treatment options. Seeing that leukocyte stiffness decreases when exposed to certain drugs in order to travel through vessels quicker, this yields a

basis for this experiment since hematopoietic stem cells need to mobilize from the bone marrow. Therefore, this paper is significant because it sets up the possibility of research.

Research Goals and Objectives

Overall, the current study will address the gap in understanding of hematopoietic stem cell mechanical properties during mobilization under normal conditions as well as under treatment from AMD3100 and G-CSF. The current study will determine mechanical properties of HSCs using microfluidic models, various microscopy techniques, and flow cytometry to confirm findings. By investigating the mechanical properties of HSCs during mobilization in the presence of G-CSF as well as AMD3100, clinical significance can lead to further studies as well as alternative mobilization techniques for use with HSC transplantation for patients with hematological malignancies as well as bone marrow failure. In addition, a purely mechanical result in increased mobilization will be observed as opposed to the currently accepted mechanisms of G-CSF and AMD3100, respectively. Ultimately, a mechanical understanding of HSCs will allow for a greater understanding of hematopoiesis and mobilization.

CHAPTER 3

MATERIALS AND METHODS

In order to observe and characterize mechanical properties associated with the stiffness of HSC, biophysical flow cytometry was used (Rosenbluth, Lam, & Fletcher, 2008). By using the microfluidic-based biophysical flow cytometry, single cell HSC stiffness during mobilization into the peripheral blood was characterized by the transit time of each cell through the capillary bed model of the microfluidic device under *in vitro* conditions.

Biophysical Flow Cytometers

Biophysical flow cytometry devices (Figure 1) with 5.9 ± 0.8 μm-wide channels were fabricated following the method outlined by Rosenbluth, Lam, & Fletcher. In short, PDMS mixed from a Sylgard 184 Silicone Elastomer Kit was molded to a mask formed via typical microfabrication techniques and then cut out. Prior to being bonded to a 22 x 40mm cover glass slide (Corning) using a plasma cleaner (Harrick Plasma PDC-32G), inlet and outlet holes were punched into the device. The microfluidic device was then blocked with 10% Bovine Serum Albumin (BSA) (Sigma-Aldrich) in 1x Phosphate Buffered Saline (PBS).

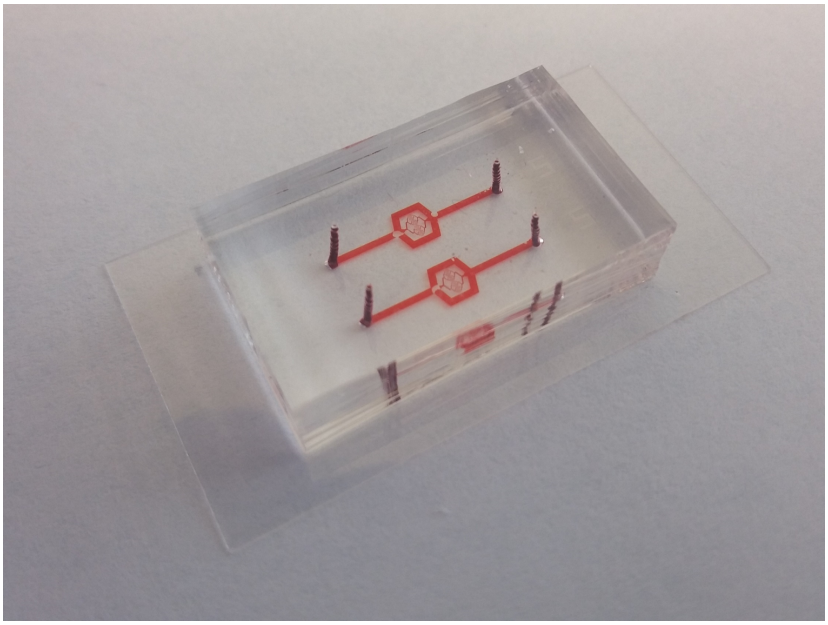

Figure 1. Biophysical flow cytometry device. The microfluidic capillary bed model consists of channels that are 5.9 ± 0.08 μm wide, which are smaller than the average diameter of hematopoietic stem cells (HSCs). Using a mold developed via soft lithography, Polydixmethylsilane (PDMS) was poured onto the mold and cured at 60 ˚C. The device was then cut from the mold, inlet and outlet holes were poked through the device and then bonded to coverslips using a plasma cleaner. At least 1 hour before use, the device was blocked using a 10% bovine serum albumin in phosphate buffered saline solution.

Hematopoietic Stem Cells

Hematopoietic stem cells from mice were provided by the Cheng-Kui Qu Lab within the Cancer Cell Biology Program at the Winship Cancer Institute at Emory University. HSCs were treated with AMD3100 octahydrochloride (Sigma) and G-CSF human, recombinant (Sigma).

Low Cell Count BFC Protocol

Due to the availability and concentration of mice HSCs, the protocol for conducting the flow cytometry experiments outlined by Rosenbluth, Lam, & Fletcher could not be followed; therefore, a low cell count protocol for biophysical flow cytometers was developed. About 2,000 HSCs, a 2 μL dilution from a 1 x 10^6/1 mL sample of HSCs, were loaded into a pipet tip with microcapillary for loading gels (VWR)

and then inserted into the inlet of the microfluidic device. A 5mL Leur-Lok syringe (BD) with an attached 20GA x ½" needle (Techcon Systems) was then attached to the outlet of the device with thick tubing and then #30 AWG thin wall tubing (Cole-Parmer Instrument Company) and then locked onto a syringe pump (Harvard Apparatus PHD Ultra). Cells were then withdrawn through the device via the pump at a flow rate of 10 μL/min and recorded via bright field microscopy with a 20x 0.45A air objective on a Nikon Eclipse TE2000-U and Qimaging Retiga EXi camera along with NIS Elements.

Data Analysis

Transit times were then calculated manually from obtained flow video using NIS Elements (Figure 2). Differences in relative entrance and exit frames were calculated and then divided by the overall frames per second (FPS) to obtain respective transit times. Mann-Whitney tests at a 5% significance level were then conducted using MATLAB to determine statistical significance between respective transit times between untreated and treated cells. Relative frequency within specific transit times were then plotted for both treated and untreated cells as well using Origin.

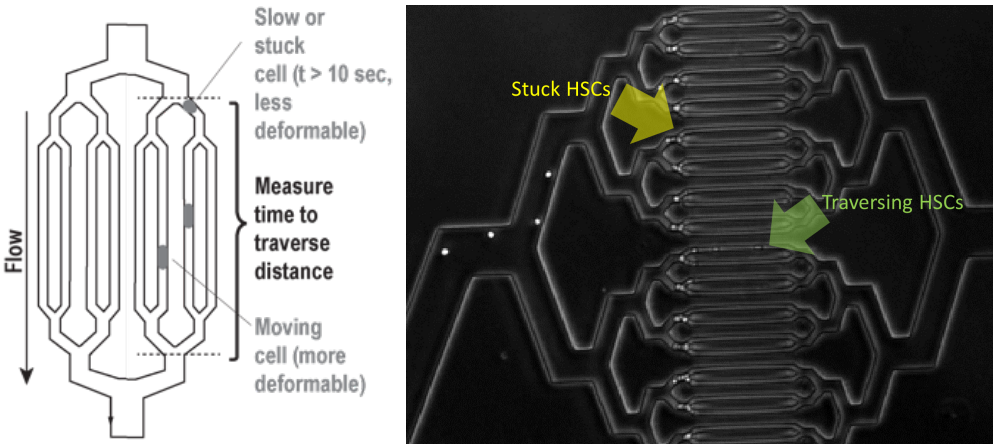

Figure 2. Detailed view of the microfluidic capillary bed model. The smallest channels have a width of 5.9 ± 0.08 μm. Once flow is applied by withdrawing HSCs through the device using a syringe pump, cells traverse through the microfluidic capillary bed model in the direction of flow. Stuck cells can be identified as those that remain at the inlet of the smallest channel for a period greater than 10 seconds and traversing cells as those that are moving through the channels. Brightfield videos of the experiment were recorded and then manually analyzed to determine transit times over a set distance, indicated above. A faster transit time would therefore indicate a more deformable, or softer, HSC suggesting a change to their mechanical properties.

CHAPTER 4

RESULTS AND DISCUSSION

Results

To determine whether mechanical properties of HSCs are altered during treatment to mediate mobilization, biophysical flow cytometry experiments were conducted to eliminate confounding variables that may exist otherwise. The microfluidic system is a capillary bed model within the microvasculature with channel widths that are smaller than the average diameter of HSCs; changes in mechanical properties are determined by transit time changes due to deformation of the HSCs.

Given the difficulty in obtaining HSCs for this research, results were obtained from experiments using AMD3100 as the only treatment, leaving G-CSF yet to be tested as a treatment condition. Flow videos were recorded for both untreated negative control HSCs and HSCs treated with AMD3100 at a concentration of 40μM. Upon manually calculating relative transit times for both the control HSCs and AMD3100 treated HSCs, a Mann-Whitney test was conducted and found to yield a $p < 0.05$ (Figure 3). Additionally, HSCs treated with AMD3100 had a higher frequency of lower transit times through the microfluidic channels versus control where a larger portion of HSCs had longer transit times. HSCs treated with AMD3100 were observed to traverse the channels with a significantly faster transit time, with a smaller portion of cells becoming stuck within the channels when compared to untreated HSCs.

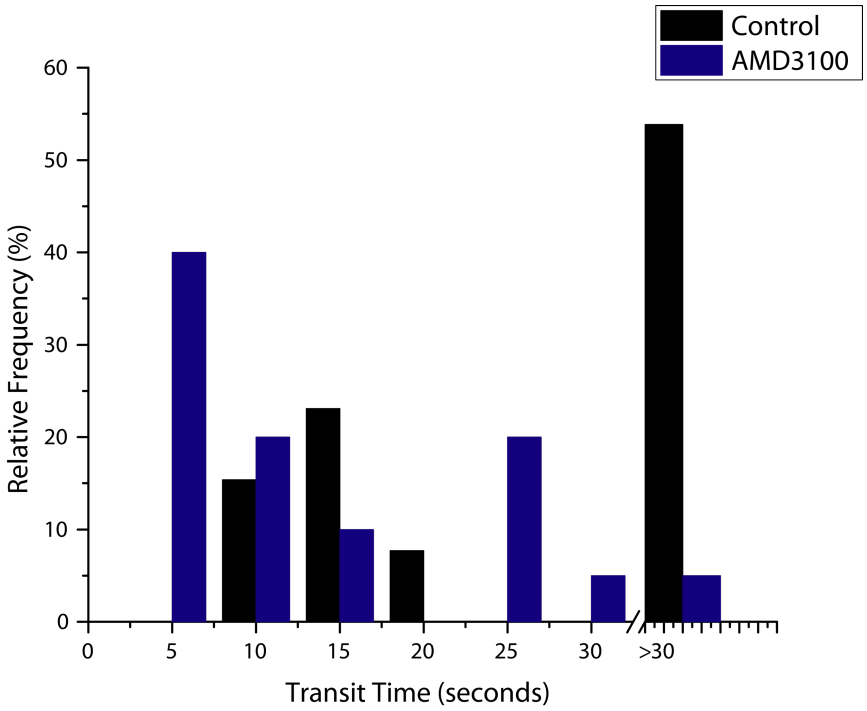

Figure 3. Histogram comparing transit times of HSCs. Upon obtaining relative transit times for both control and AMD3100 conditions, a Mann-Whitney test was conducted using MATLAB. Compared to control, AMD3100 treated HSCs traversed the microfluidic capillary bed channels significantly faster with $p < 0.05$ and were less likely to be stuck at the inlet of the capillary channel. This indicates that AMD3100 mediated cellular softening due to observed deformation through faster transit times and a decrease in stiffer stuck cells.

Discussion

This research shows that HSCs treated with hematopoietic growth factor cytokines, specifically AMD 3100, have shorter transit times when traversing through the microfluidic channels. Shorter transit times indicate that the HSCs are becoming less stuck within the channels and are traversing through the channels faster. As a result, this indicates changes to the mechanical properties of the HSCs as the cells are becoming softer through a decrease in the cellular stiffness. Additionally, since changes to the stiffness of HSCs are observed when treated with approved mobilizing agents, this may shed light to the initial mechanism by which HSCs are mobilized into the peripheral blood through changes in the cells' mechanical and physical properties.

Similarly, the cytoskeleton of leukocytes has previously been shown to soften due to glucocorticoids, like dexamethasone, and catecholamines, like epinephrine. Using a similar biophysical flow cytometry method, additional microfluidic systems, as well as computational modelling, decreased cell stiffness and increased demargination have shown to directly increase clinical CBC (complete blood count). This parallel between leukocyte and HSC softening may indicate that softening of the cytoskeleton of blood cells may be a critical aspect in their trafficking mobilization in the peripheral blood.

CHAPTER 5

CONCLUSION AND FUTURE WORK

AMD3100 has been approved by the FDA to increase HSC mobilization, but the mechanical properties of HSCs have yet to be studied until now. Through biophysical flow cytometry using microfluidic systems, mechanical properties of HSCs have been observed to be altered through cellular softening when treated with a mobilizing agent. This indicates that during mobilization, HSCs may undergo changes to the cytoskeleton which allows for their mobilization into the peripheral blood. By investigating the mechanical properties of HSCs during mobilization, clinical significance can lead to additional studies and the discovery of novel mobilization techniques for use with HSC transplantation for patients with hematological malignancies.

While this research has identified HSC softening due to AMD3100, a mobilizing agent, additional studies will need to be conducted for G-CSF treatments as well. Additionally, atomic force microscopy will also need to be conducted to obtain numerical cell stiffness values for control HSCs, AMD3100, and G-CSF treated HSCs. These values will then allow for the direct statistical comparison of how cellular stiffness is altered and to what degree by the hematopoietic growth factor cytokines. Finally, human subject testing through ingestion of AMD3100 and G-CSF will need to be conducted to determine the possible clinical implications and confounding factors that exist in vivo.

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