Effect of Crizanlizumab on Blood Cell Adhesion in Sickle Cell Disease: A Standardized Microfluidic Assessment via the Endothelium-on-a-Chip

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INTRODUCTION

- Chronic upregulation of P-selectin on blood cells and the endothelium leads to abnormal red blood cell (RBC) adhesion to endothelial cells, significantly contributing to vaso-occlusive crises (VOCs), which are a major cause of morbidity and mortality in patients with sickle cell disease (SCD). Crizanlizumab (a.k.a. SEG101) is a humanized anti-P-selectin monoclonal antibody and has recently been approved by the Food and Drug Administration to reduce the frequency of VOCs in SCD patients.
- There is currently no existing feasible clinical *in vitro* model for monitoring the patient-specific effects of Crizanlizumab on cellular adhesion, which we would argue is largely due to the lack of a universally accepted, standardized physiologic flow-based adhesion assay with which to measure blood cell adhesion to human endothelial cells. Utilizing such an *in vitro* assay would greatly help visualize cellular adhesion before and after therapeutic interventions and may reveal patient-specific responses to combination therapies.
- To this end, we have developed a standardized endothelialized microfluidic platform: **Endothelium-on-a-chip**, for *in vitro* assessment of the effect of Crizanlizumab on patient-specific RBC adhesion to heme-activated human endothelial cells. We have previously shown that RBC adhesion to heme activated endothelial cells may correlate with a more severe disease phenotype.

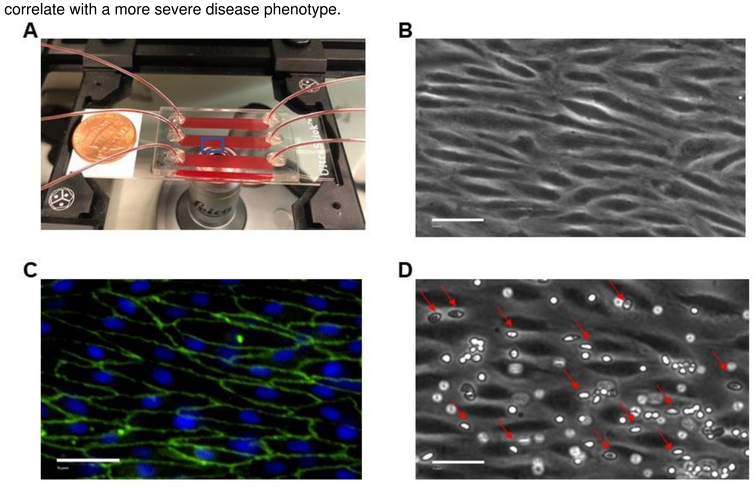

Figure 1. The proposed microfluidic system functionalized with HUVECs. (A) Endothelium-on-a-chip set-up. The phase-contrast **(B)** and fluorescent **(C)** images of HUVEC demonstrate a confluent layer of cells aligned with flow (Hoechst blue, CD144 green). **(D)** RBCs adhesion to Heme activated HUVECs. Microscope images at 10X. Scale bare 50um

MATERIALS & METHODS

- Microfluidic channels were fabricated via a lamination technique using laser micro-machined components. The device assembly was performed by placing a laser-cut double sided adhesive (DSA) film in between an acrylic top cover, that includes the inlet and outlet ports, and a glass microscope slide (**Fig.1A**). The height of the microchannels was determined by DSA thickness, which was 50 µm mimicking the size scale of human post-capillary venules.
- To prepare endothelialized microchannels, Human Umbilical Vein Endothelial Cells (HUVECs) were cultured and seeded into fibronectin-functionalized microchannels at a density of $8x10^6$ cells/mL and incubated in static conditions for 2 hours at 37°C and 5% CO_2.
- The endothelialized microchannels were then cultured under flow (~15 dyne/cm^2) for 48-hours at 37°C and 5% CO_2 prior to adhesion experiments. **Figure 1 B** and **C** show the HUVEC aligned in the channel.
- Adhesion experiments were performed for two different endothelial activation durations: 4 hours (long-term) and 30 minutes (short-term). For long-term activation, HUVECs were treated with 40 µM of heme for 4 hours +/- 100 µg/ml Crizanlizumab for 1 hour followed by injection of blood samples through the microfluidic channels. For short-term activation, blood samples were supplemented with 40 µM heme +/- 100 µg/ml Crizanlizumab and injected through the microfluidic channels for 15 minutes. Thereafter, non-adherent RBCs were rinsed via either only 40 µM heme-containing basal medium or 40 µM heme- and 100 µg/ml Crizanlizumab-containing basal medium for another 15 minutes.
- Whole blood samples were collected from 19 subjects with SCD (18 HbSS and 1 HbSC) in EDTA vacutainers. RBCs were isolated via centrifugation from whole blood and then resuspended in basal cell culture medium at a hematocrit of 20% buffered with 10 mM of HEPES.
- For the long-term activation experiments, isolated RBCs were directly injected over endothelial cells. For the short-term activation experiments, heme and Crizanlizumab were added into this RBC suspension prior to injecting the samples.
- Detachment experiments were also conducted in this study to determine whether Crizanlizumab would impact already established adhesive events between RBCs and endothelial cells (a.k.a. "crisis-resolving" effect). To do so, RBCs were first allowed to adhere to heme-activated HUVECs (for 15-min) and then the microchannels were rinsed via either a heme- or both heme- and Crizanlizumab-containing solution for another 15 minutes at 1 dyne/cm^2. We then quantified the number of adherent RBCs at min=0 and min=15.

RESULTS AND DISCUSSION

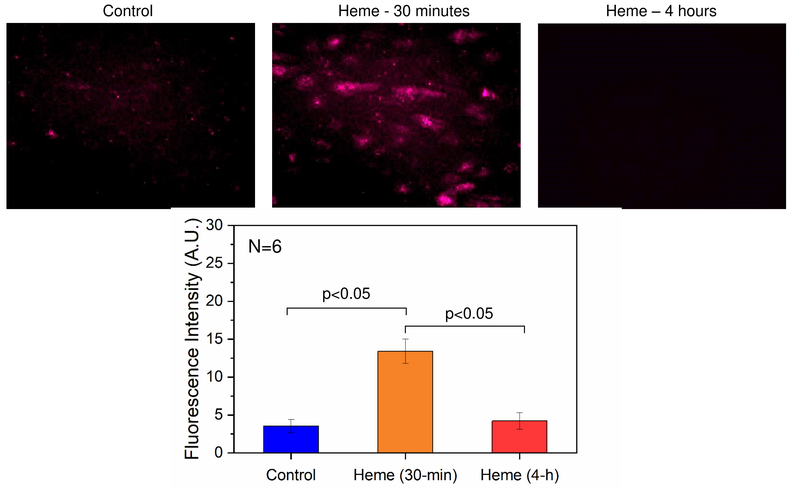

Figure 2. P-selectin surface expression on HUVECs in response to heme activation. Short term heme activation led to a statistically significant increase in surface P-selectin presence while increasing the activation duration to 4 hours lowered the amount of surface P-selectin expression. Fluorescence intensity was quantified using ImageJ.

- Short term (30-min) heme activation significant increase endothelial surface P-selectin expression based on our immunofluorescence studies as shown in **Figure 2.** Long term (4-hour) heme activation brings the surface P-selectin expression down to the basal level while we observed statistically significant elevated E-selectin and ICAM-1 expression levels following a 4-hours heme activation (data not shown).
- 4-hours heme activation of HUVECs resulted in statistically significant elevated RBC adhesion compared to baseline while adhesion levels were heterogenous among the patient population (**Fig. 3A**, 1671±522 vs 17±4, p<0.05).
- Treatment of 4-hours heme-activated HUVECs with Crizanlizumab did not statistically significant decrease RBC adhesion (**Fig. 3A**, 1170±413 vs 1671±522, p>0.05), while we observed lower RBC adhesion to Crizanlizumab treated HUVECs for certain subjects (**Fig. 3B**).
- Crizanlizumab treatment statistically significant reduced the number of adherent RBCs to 15-minute heme-activated HUVECs (**Fig. 3C**, 135±40 vs 1513±617, p<0.05). Crizanlizumab treatment of HUVECs was able to decrease RBC adhesion for each patient who was tested while certain patients did not have considerable baseline adhesion levels (**Fig. 3D**).
- As shown in **Figure 3E**, perfusion of heme-containing wash medium supplemented with Crizanlizumab resulted in higher RBC detachment rates compared to heme-only medium for all three subjects. On average, the percentage of remaining RBCs after a 15-minute wash was only 10% when the heme-containing solution included 100 µg/ml Crizanlizumab. By contrast, more than half of the RBCs still remained in the microfluidic channels without the addition of Crizanlizumab.
- In **Figure 3F** the HUVECs were activated with TNFα (20 ng/ml) for 4 hours previous the short-term (30 minutes) treatment with heme in presence or not of the Crizanlizumab. Crizanlizumab decrease the adhesion of RBCs to the TNFα and heme activated HUVECs.
- Our results show that the magnitude of inhibition of RBC adhesion to HUVECs with Crizanlizumab is influenced by the duration of heme-activation (4 hours vs 15 minutes). This is likely due to variable levels of different adhesion molecules on acute or chronically activated HUVECs. These preliminary results suggest that the Endothelium-on-a-chip, as partner in novel therapeutic studies, could help monitoring dynamics of targeted therapies in SCD patients during drug development and in clinical trials.

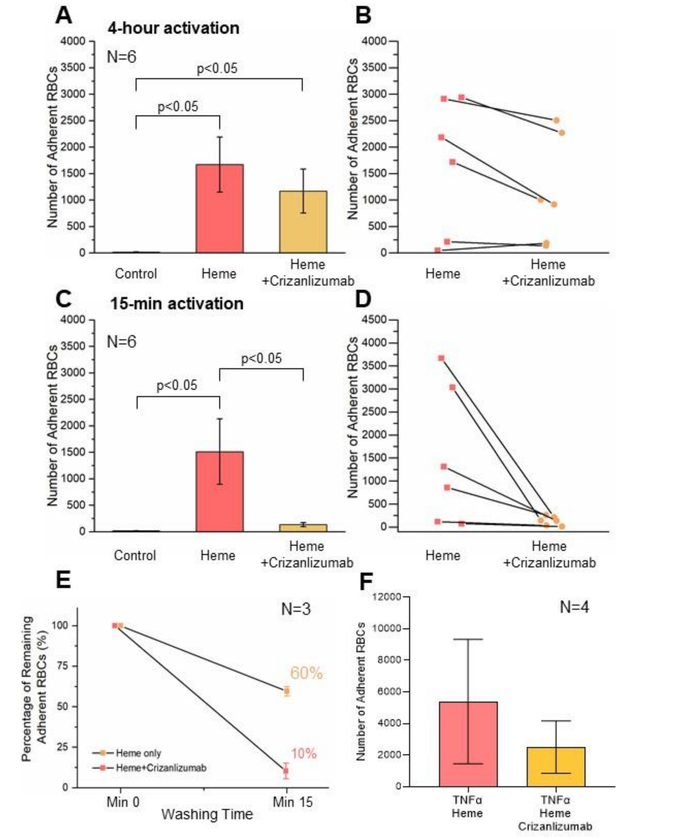

Figure 3. Effect of Crizanlizumab on SCD RBC adhesion to heme-activated HUVECs. (A) 4-hours heme activation statistically significant increases RBC adhesion to HUVECs while pre-incubation of heme-activated HUVECs via Crizanlizumab has a mild effect in lowering the RBC adhesion levels. **(B)** RBC adhesion to 4-hours heme-activated HUVECs has a strong subject-specific dependency. **(C)** Crizanlizumab treatment of 15-minutes heme-activated HUVECs resulted in statistically significant reduced RBC adhesion levels. **(D)** Inhibition of RBC adhesion was largely independent of subject-specific baseline adhesion levels. **(E)** RBCs were first allowed to adhere to 15-min heme-activated HUVECs and then rinsed with a solution containing either only heme or both heme and Crizanlizumab. The Crizanlizumab containing solution resulted in 90% of the adherent cells to detach after a 15-minute rinse while 60% of the RBCs remained adherent after a 15-minutes rinse with the solution that did not contain Crizanlizumab. (**F**) The short-term heme activation was preceded by 4-hours treatment with TNFα. Crizanlizumab reduce the number of adherent RBCs to the HUVECs pretreated with TNFα and heme. For all experiments, we used the following concentrations heme 40 µM, Crizanlizumab 100 µg/ml and TNFα 20 ng/ml. P-values were based on student's t-test. Error bars represent the standard error of the mean (SEM).

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