An ex vivo Platform to Evaluate Compound Effects on Erythroid and Megakaryocyte Development

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Introduction

Thrombocytopenia and anemia are on-going problems in drug development. Small animal in vivo models, which are commonly used to assess thrombocytopenia and anemia risk at the development stage, often do not translate well into clinical trials. Although progenitor assays, measuring the primitive cells in the bone marrow are excellent at assessing toxicity with some drug classes, it appears that some of these primitive cell populations may be "spared" by the drugs, whereas their progeny – the more mature cells in the blood system- are more susceptible to the toxic effects. We have previously shared data on a megakaryocyte platform that assesses the proliferation and differentiation of cells from early progenitors to mature platelets based on CD41 expression (SOT 2015). This study extends our findings to the erythroid lineage, looking at red blood cell development from primitive CD34+ cells to mature red blood cells. Additionally, we evaluated a method to expand progenitor cells using an aryl hydrolase antagonist, which could provide suitable cell numbers for drug screening using these platforms, and assessed whether the phenotypic profiles of the expanded cells over the course of the study were similar to their non-expanded counterparts.

Objective

The initial goal of this work was to create assays that can accurately predict the risk of severe anemia associated with investigational biotherapeutics. To be considered predictive, the assay should, as a starting point, recapitulate in vitro the known in vivo biology of red blood cell development in a biologically relevant, reproducible and quantitative method. An additional goal was to assess the expansion of multipotent hematopoietic progenitor cells using an aryl hydrolase (AhR) antagonist, and to compare the kinetic proliferation and differentiation profiles of the megakaryocytes and platelets in these populations as compared to their non-expanded counterparts.

Materials and Methods

Erythroid Assays

CD34+ cell populations derived from normal human bone marrow (NorCal Biologics, Placerville, CA) and processed at ReachBio were cultured in medium containing SCF, IL-3 and EPO (added initially or 7 days later) in a 96 well format over a 21 day period in the presence and absence of AZT (10 µM), Imatinib (1.0 µM) and dexamethasone (1.0 µM). At various times, a cocktail of antibodies containing DAPI, CD34, CD45 and CD235a (glyA), (Beckman-Coulter, Brea, CA) were used to stain the cultured cells. Flow cytometry analysis was then performed using a cytoFLEX® flow cytometer. Additionally, these drugs were added to a methylcellulose based medium known to support erythroid and myeloid progenitors to assess clonal growth, and plated in 35 mm dishes. The cultures were incubated for 14 days and then scored microscopically.

CD34+ Cell Expansion Assays

Two lots of bone marrow CD34+ cells and 2 lots of cord blood CD34+ cells were expanded in the presence of an aryl hydrolase antagonist (Fares, 2014). On day 7, the cells were counted and assessed for CD34+ expression by flow cytometry. A sample of cells (50,000 /mL) were replated into the complete medium and the process of cell counting and FACS analyses performed again on day 14.

The expanded bone marrow cells were set up in our megakaryocyte platform assay as described previously (SOT 2015). On days 7 and 10 a cocktail of antibodies containing DAPI, CD34-PE and CD41-FITC (Beckman-Coulter, Brea, CA) were used to stain the cultured cells. Flow cytometry analysis was then performed using a cytoFLEX® flow cytometer.

Results: Erythroid Assays

The test compounds had various effects in both the colony assay (evaluating red blood cell progenitors) and the liquid culture assay (evaluating red blood cell development). AZT significantly inhibited the BFU-E as well as the development of the red blood cells. Dexamethasone had no inhibitory effect on the BFU-E and appeared to slightly augment red blood cell development on day 14. Imatinib had no effect on the BFU-E, though significantly inhibited red blood cell development at both time points (Table 1 and Figure 1).

Red blood cells expand well over 14 days in the presence of IL-3, SCF and where Epo was added at the initiation of the experiment or on day 7. In the presence of Epo, there were significantly more red blood cells at day 7 when compared to cultures without Epo. However at day14, there were increased numbers of total viable cells and comparable numbers of red blood cells in the cultures where Epo was added on day 7 (Figure 1). The toxic effects of AZT and Imatinib on red blood cell development are clearly visible on the FACS profiles (Figure 2), whereas it appears that Dexamethasone may be delaying red blood cell development on day 7 (CD45+ CD235a+) but, by day 14, the red blood cells have clearly matured (Figure 2) (CD45- CD235a+) .

Results: Cell Expansion and Megakaryocyte Assays

Both cord blood and bone marrow CD34+ cells expanded in the presence of an AhR antagonist, with significantly higher CD34+ cell percentages at days 7 and 14 as compared to traditional expansion methods with cytokines in the absence of an AhR antagonist. There appeared to be an inverse relationship between the expansion rate and the percentage of CD34+ cells at 14 days (Table 2). We compared the profile of megakaryocyte development on day 7 between unexpanded CD34+ bone marrow cells and those expanded with an AhR antagonist (Figure 4). The relative percentages of the early, mid and late megakaryocytes as determined by CD41 expression differed between the expanded and non-expanded cell populations, with the expanded cells having less differentiation to mature megakaryocytes (Figure 4). We performed a similar comparative study looking at red blood cell development from non-expanded and AhR-expanded CD34+ cells. Once again, the relative percentages of mature red blood cells differed between non-expanded and expanded CD34+ cells (Figure 4).

	Erythroid CFC	Myeloid CFC
Solvent Control	28 +/- 3	40 +/- 4
AZT (10 µM)	8 +/- 2**	18 +/- 5*
Dexamethasone (1.0 µM)	31 +/- 4	40 +/- 4
Imatinib (1.0 µM)	26 +/- 5	27 +/- 3

* $p < 0.01$, ** $p < 0.001$

Table 1. Effect of compounds on erythroid and myeloid progenitors using colony forming cell assays

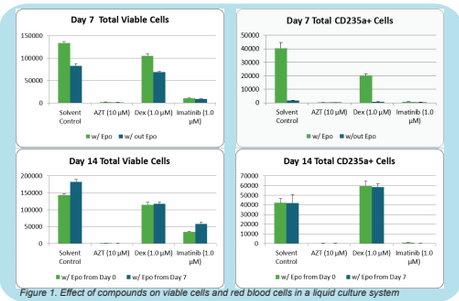

Figure 1. Effect of compounds on viable cells and red blood cells in a liquid culture system

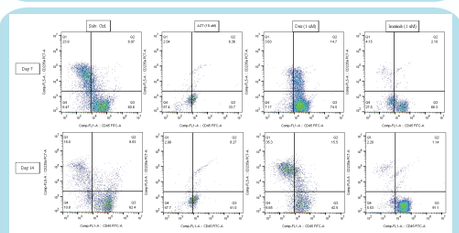

Figure 2. Typical profiles of red blood cell development in the presence of test compounds

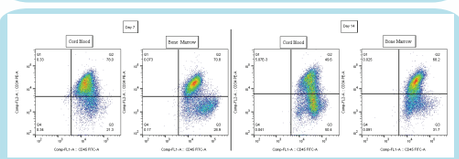

Figure 3. Representative CD34 percentages from expanded cord blood and bone marrow samples

Sample*	Day 7 Expansion	Day 7 CD34+ Percentage	Day 14 Expansion	Day 14 CD34+ Percentage
CB lot # 0120816	7.3x	78	257x	50
CB lot # 0120809	35.2x	72	1007x	38
BM lot # 0120908	6.9x	71	57x	68
BM lot # 0120112	7.5x	75	350x	44

* Purity of CD34+ cells was greater than 94% in all start samples

Table 2. Expansion numbers and CD34 percentages from cord blood bone marrow samples.

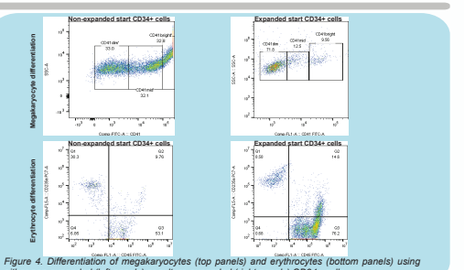

Figure 4. Differentiation of megakaryocytes (top panels) and erythrocytes (bottom panels) using either non-expanded (left panels) or culture-expanded (right panels) CD34+ cells.

Conclusions

CD34+ bone marrow cells can differentiate along the erythroid lineage and generate mature red blood cells (CD45- CD235a+). The expansion of red blood cells at day 14 is comparable whether Epo is added at the initiation of the study or after 7 days.

AZT at 10 µM and Imatinib at 1.0 µM significantly inhibited mature red blood cell development. The CFC assay confirmed that AZT at 10 µM significantly inhibited the red blood cell progenitors (BFU-E), which explained the reduction in mature red blood cells. With Imatinib, the significant inhibition of mature red blood cells was not due to an effect on the BFU-E population suggesting that Imatinib has an effect on a more mature red blood cell precursor.

Dexamethasone at 1.0 µM had no effect on the erythroid progenitor (BFU-E) population and at day 14 had increased red blood cell numbers as compared to solvent controls.

Progenitor assays in conjunction with differentiation assays can help elucidate the mechanism of action of test compounds.

The presence of an AhR antagonist in the expansion medium allowed for significant increases in the CD34+ cell number but these expanded cells , when subjected to various differentiation protocols, did not show the same profiles in terms of megakaryocyte or erythrocyte maturation as compared to their non-expanded counterparts. Although promising, AhR antagonist expanded CD34+ cells may not be substituted for non-expanded CD34+ cells in differentiation assays currently.

References

Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. I Fares, J Charaoui, Y Gareau et al. Science (2014)

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