Ex vivo expansion and differentiation of erythroblasts: from culture dishes to stirred bioreactors for the production of red blood cells

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ABSTRACT

Transfusion of donor-derived red blood cells (RBCs) is the most common form of cell therapy. Nevertheless, it faces challenges such as emerging blood-borne diseases, and supply limitations; for instance, in low-income countries, or for chronically transfused patients requiring special blood groups. Production of cultured RBCs (cRBCs), in which erythroid precursors (erythroblasts) are cultured from hematopoietic progenitors and subsequently differentiated into transfusion-ready erythrocytes, is a potential alternative. However, the large number of cRBCs required for a single transfusion unit requires major innovations in the culture process.

Stirred bioreactors are conventionally used for other mammalian cell cultures. In this culture system, turbulence is a critical parameter, due to the reported detrimental effects of shear stress and energy dissipation due to mixing in the growth of some mammalian cell lines [1]. Furthermore, the bioreactor materials can also influence the performance of the cell cultures, as the conventionally used stainless steel can leach metal ions that impact cell growth and viability [2]. The aim of our work is to scale-up the expansion and differentiation of erythroblast for the production of cRBCs using stirred bioreactors. In addition, we aim to compare the performance of two stirred bioreactor types (a conventional glass & stainless steel autoclavable bioreactor, and single-use plastic bioreactors).

We have successfully performed the expansion of erythroblast cultures using Applikon MiniBio 500 mL and single-use Applikon AppliFlex ST 0.5 L stirred tank bioreactors, following a repeated batch cultivation strategy. Erythroblasts produced in these systems have shown the same proliferation potential and similar expression of erythroid surface markers (CD235, CD71) as those cultured in conventional static conditions, while maintaining a high viability during the cultivation, suggesting that the potential negative effects of excessive shear rates and turbulence have been avoided.

In addition to proliferation, the differentiation of cultured erythroblast into mature reticulocytes was also tested in the stirred bioreactors. Slightly lower cell yields were observed in the stirred cultures compared to static conditions, in agreement with previous reports on the effect of shear stress on the differentiation dynamics of erythroblast cultures. During erythroblast differentiation, cells lost expression of CD71 and CD49d at a comparable rate to cells kept under static conditions (culture dishes). These results show that the expansion and differentiation of erythroblast cultures is feasible in stirred bioreactors, and indicate that the single-use AppliFlex ST platform is adequate for the transition to GMP production of cRBCs.

MATERIALS AND METHODS

For expansion culture experiments, erythroblasts were derived from peripheral blood mononuclear cells under static conditions, as previously described [3]. After 8 days, emerging early erythroblasts were transferred to the stirred bioreactors (MiniBio 500 mL and AppliFlex ST 0.5 L). For expansion cultures, a repeated batch cultivation was followed for 6 days in both bioreactors and in culture dishes in order to compare the performance between them. Harvesting and medium refreshment was performed daily, maintaining a cell density of 0.7-1.0×106 cells/mL and a culture volume of 250 mL.

For differentiation cultures, peripheral blood mononuclear cells were cultured until a pure erythroid culture was obtained (day 8), followed by 3 extra days of culture to obtain an adequate number of erythroblasts to inoculate the bioreactors. These day 11 erythroblasts were then transferred to the stirred bioreactors (MiniBio 500 mL or AppliFlex ST 0.5 L) and were cultured in batch mode.

An IMDM-based defined medium was used for the cell cultures, supplemented with growth factors depending on the culture stage (human stem cell factor, dexamethasone and erythropoietin for expansion; erythropoietin, plasma, transferrin and heparin for differentiation) [3]. Dissolved oxygen (dO_2) concentration was kept in all cultivations at 40% (air saturation, at 37°C and 1 atm) via air sparging. pH was maintained around 7.5 either by including 5% CO_2 in the air stream used to control dO_2, or by separately sparging pure CO_2. Temperature was kept at 37°C. Stirring was performed using a down-pumping marine impeller with a speed of 200 rpm in both bioreactors.

Cell concentration was determined by cell counter (Beckman Coulter Counter, or CASY Model TCC) using the number of cells with a diameter larger than 6.0 µm for expansion cultures or larger than 5.0 µm for differentiation cultures.

Viability, expression of differentiation markers (transferrin receptor CD71, glycophorin A CD235a, integrin alpha 4 CD49d), and enucleation efficiency was monitored by flow cytometry.

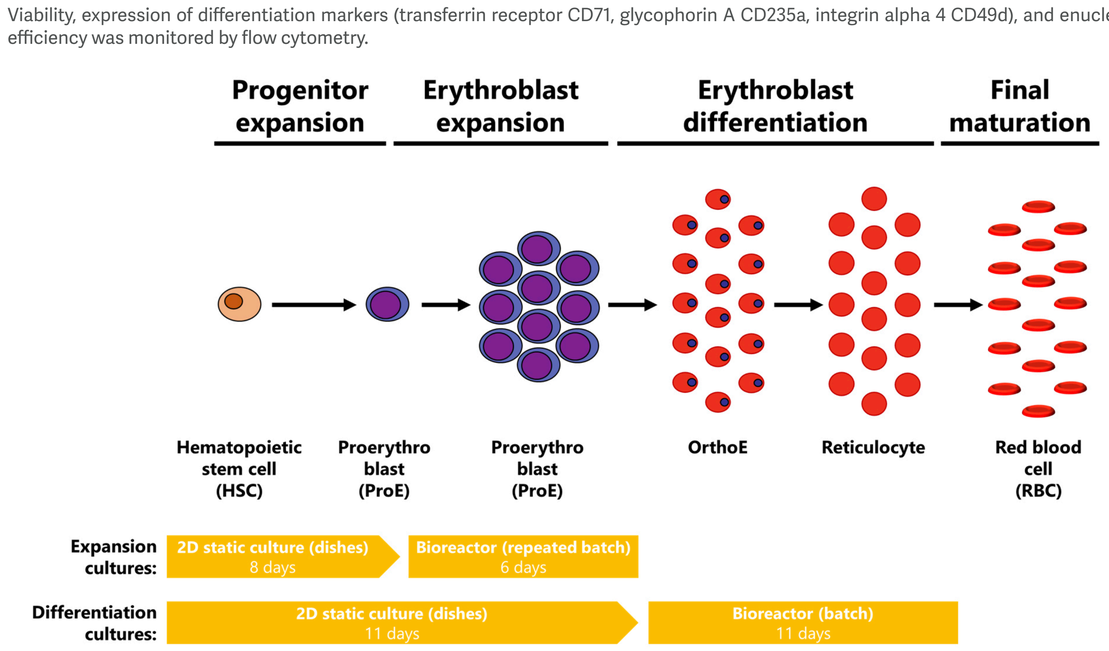

Figure 1. Scheme of the experimental design followed for the bioreactor cultures. Peripheral blood mononuclear cells, containing hematopoietic stem cells, were cultured for 8 days under static conditions (culture dishes). At this time, proerythroblasts represented the majority of the cells. This proerythroblast-enriched culture was transferred to the stirred bioreactors. In the case of differentiation cultures, proerythroblasts were kept under static culture conditions for 3 extra days (total = 11 days), transferred to the stirred bioreactor, and kept for 11 days.

RESULTS

Expansion

The expansion of erythroblasts was carried out in two types of stirred bioreactors (MiniBio 500 mL and AppliFlex ST 0.5 L) following a repeated batch operating mode. Briefly, cells were seeded at 1×106 cells/mL in expansion medium. After 24h of culture, cells were reseeded at 1×106 cells/mL using fresh expansion medium. The expansion in both systems was comparable to cultures kept under static conditions (culture dishes), as shown in Figure 2). Cumulative cell number was calculated using daily cell concentration measurements and the cumulative dilution factor due to the harvesting/feeding cycles of the repeated batch operating mode.

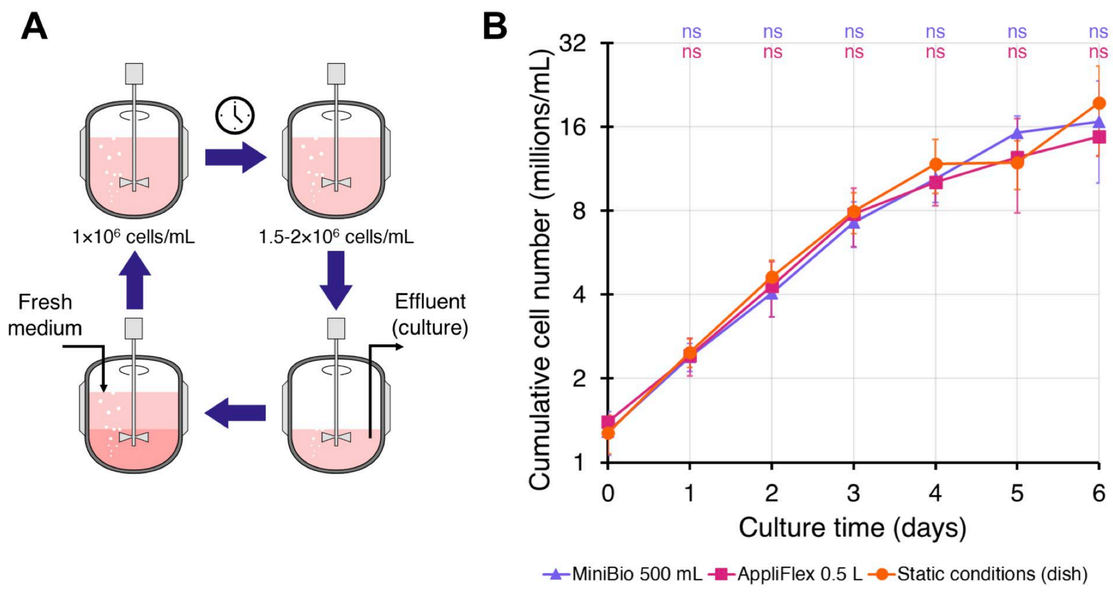

Figure 2. (A) Repeated batch cultivation strategy used for the expansion of erythroblasts in stirred bioreactors. (B) Proliferation potential of erythroblasts in expansion conditions is comparable in stirred bioreactors and static conditions. Data is displayed as mean ± SD (n≥3). Significance is shown for the comparison with the static cultures (Student's t-test; ns = non-significant, * = p-value < 0.05, ** = p-value < 0.01).

To assess the state of the cultured erythroblasts in our cultures, we measured the expression of the surface markers CD235a and CD71 by flow cytometry (Figure 3A). In the expansion phase we aim to maintain cultures in the CD71+ CD235mid/high stage (gates P2 and P3), while keeping the number of cells undergoing spontaneous differentiation (gate P4) at a low level. Erythroblast expanded in MiniBio 500 mL of AppliFlex 0.5 L bioreactors have similar expression of the erythroid surface markers CD235a and CD71a after 6 days of culture compared to static conditions. Slightly lower numbers of spontaneously differentiated cells (CD235a+ CD71+) were observed in both reactors compared to culture dishes. Terminal viability, measured as number of cells that are negative after propidium iodide (PI) staining, was slightly higher in the AppliFlex 0.5 L bioreactor cultures compared to MiniBio 500 mL bioreactors and culture dishes (Figure 3B).

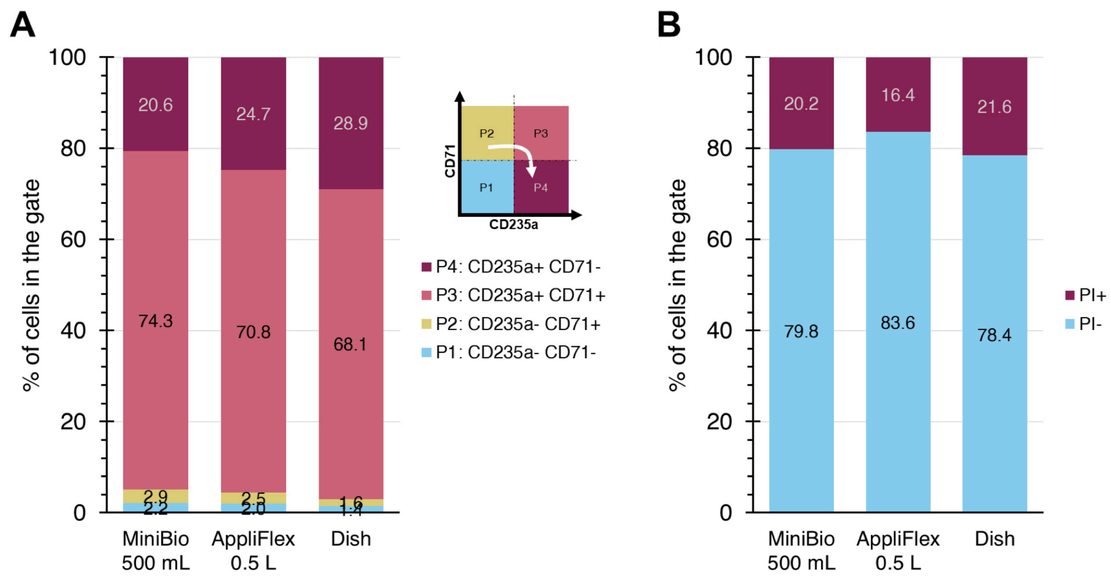

Figure 3. (A) Erythroid surface marker pattern after 6 days of culture in the stirred bioreactors and static conditions. (B) Terminal erythroblast in the dish and stirred bioreactors determined using propidium iodide (PI) staining. Data is displayed as the mean of 2 independent bioreactor runs.

Differentiation

As no statistically significant differences were observed between the two tested stirred bioreactors (MiniBio 500 mL and AppliFlex ST 0.5 L) in erythroblast expansion cultures, both reactors were assumed to also behave similarly in the differentiation stage. Similar decrease of cell size (Figure 4B), a hallmark of erythroid differentiation, was observed in stirred bioreactors compared to static condition cultures. Lower final cell yields were achieved in bioreactor cultures, although the same proliferation level was observed during the first 2 days of differentiation (Figure 4A). A similar expression of the erythroid differentiation markers CDs35a and CD71 was observed in bioreactors and static cultures (Figure 5).

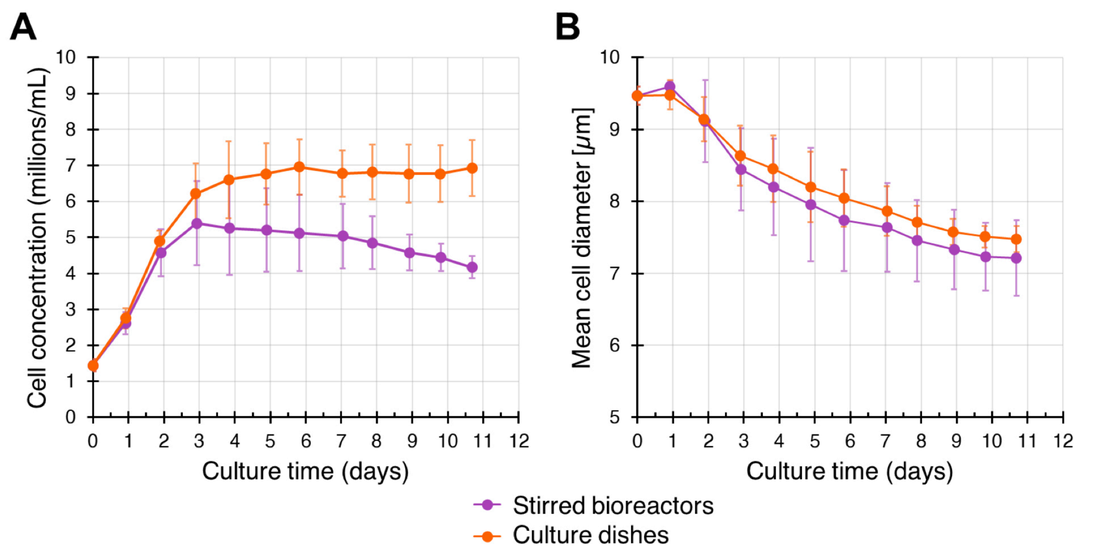

Figure 4. Erythroblasts in differentiation conditions show similar growth dynamics (A) and decrease in cell diameter (B) in stirred bioreactors compared to static conditions. Lower cell concentrations are observed for the bioreactors, in agreement with the previously described effect of mixing on differentiation cultures [4]. Data is displayed as mean ± SD (n≥3).

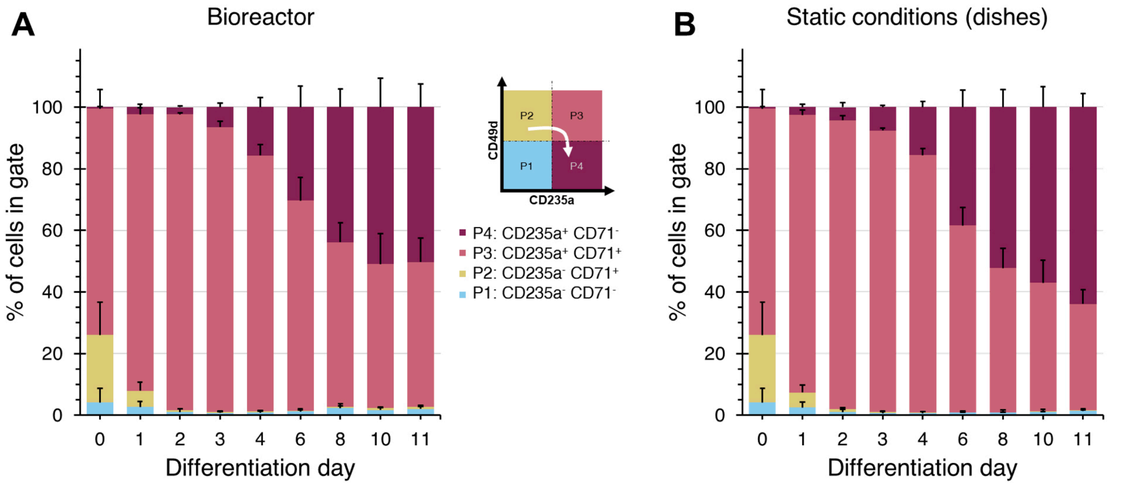

Figure 5. Erythroblast differentiated in MiniBio 500 mL or AppliFlex 0.5 L bioreactors (A) have similar expression of the erythroid differentiation surface markers CD235a and CD71 compared to static conditions (B) during the 11 days of differentiation culture.

CONCLUSIONS

Our erythroblast expansion and differentiation culture protocols have been successfully scaled up from static culture conditions to stirred bioreactors in which relevant culture parameters such as dissolved oxygen and pH are actively monitored and controlled.
Erythroblast expansion can be maintained for at least 6 days in stirred tank bioreactors (MiniBio 500 mL and AppliFlex 0.5 L) without significant difference in cell yields compared to static cultures. Expanding erythroblasts in AppliFlex 0.5 L bioreactors have a similar expression of differentiation markers compared to culture dishes, with a slightly higher terminal viability. Differentiation cultures in stirred bioreactors still show proliferation potential in the first 3 days of culture, and result in a pure CD235a+ CD49d- population after 11 days of culture.

References

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