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Simply red: A novel spectrophotometric erythroid proliferation assay as a tool for erythropoiesis and erythrotoxicity studies

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ABSTRACT

Most mammalian cell proliferation assays rely on manual or automated cell counting or the assessment of metabolic activity in colorimetric assays, with the former being either labor and time intensive or expensive and the latter being multistep procedures requiring the addition of several reagents. The proliferation of erythroid cells from hematopoietic stem cells and their differentiation into mature red blood cells is characterized by the accumulation of large amounts of hemoglobin. Hemoglobin concentrations are easily quantifiable using spectrophotometric methods due to the specific absorbance peak of the molecule's heme moiety between 400 and 420 nm. Erythroid proliferation can therefore be readily assessed using spectrophotometric measurement in this range. We have used this feature of erythroid cells to develop a simple erythroid proliferation assay that is minimally labor/time- and reagent-intensive and could easily be automated for use in high-throughput screening. Such an assay can be a valuable tool for investigations into hematological disorders where erythropoiesis is dysregulated, i.e., either inhibited or enhanced, into the development of anemia as a side-effect of primary diseases such as parasitic infections and into cyto-(particularly erythro-) toxicity of chemical agents or drugs.

1. Introduction

Erythropoiesis is one of the body's most productive cell proliferation processes, yielding an average of 2×10^{11} new erythrocytes from hematopoietic stem cells of the bone marrow every day to replace those lost to senescence and destruction [25]. A reduced erythropoietic output or the production of malfunctioning erythrocytes leads to anemia which can have severe and even fatal consequences when tissues are insufficiently supplied with oxygen [17]. Homeostasis of erythrocyte production is primarily regulated by the hormone erythropoietin (EPO), whose production is upregulated upon tissue oxygen depletion [9,30]. However, numerous factors – both exogenous (such as toxins) and endogenous (such as inflammatory cytokines) – can inhibit proliferation and/or differentiation of erythroid cells [27]. In addition, the requirement for large amounts of iron for hemoglobinization makes the process highly dependent on the availability of sufficient concentrations of transferrin-bound iron [16]. In diseases of chronic inflammation such as rheumatoid arthritis, erythropoiesis is impaired both by the direct action of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ and by upregulation of the liver hormone hepcidin, the primary regulator of iron uptake and storage, leading to a reduction in the amount of bio-available iron in circulation [10]. Many chemotherapeutic agents have also been found to induce anemia either through an inhibition of the production of EPO or a direct cytotoxic/inhibitory effect on erythroid cells [6,31].

A number of infections with parasitic agents such as *Plasmodium*, *Schistosoma*, *Leishmania* or hookworms result in anemia [22,24]. In the case of intestinal infections, this anemia is believed to be caused primarily by intestinal hemorrhage, reduced iron absorption or decreased bioavailability of iron [29]. Inflammatory responses to the infections including the secretion of proinflammatory cytokines and/or the resultant upregulation of hepcidin additionally appear to inhibit erythropoiesis, as in the anemia of chronic disease [4,27]. In the case of malaria and leishmaniasis, evidence exists that parasitic products may also directly impede erythroid proliferation and/or differentiation [13,33].

On the other hand, erythropoiesis can become dysregulated in certain myeloproliferative disorders leading to uncontrolled proliferation of erythroid cells. In the erythroleukemia polycythemia vera for example a mutation in the Janus tyrosine kinase

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JAK2 renders erythroid proliferation independent of erythropoietin and causes excessive red cell production [20,23].

In vitro methods for the generation of erythroid cells from hematopoietic stem cells derived from various sources have been established and shown to yield both high proliferation of erythroid cells and produce functional, mature, enucleated reticulocytes or erythrocytes, thus faithfully recapitulating the in vivo process [3,11,12]. In general, the differentiation process of erythroid progenitor cells and their maturation is characterized by the acquisition of specific erythroid features including particular surface markers, an exit from the cell cycle and the accumulation of large amounts of hemoglobin that is responsible for the cells' ability to bind oxygen [35,39]. A tetramer of 4 globin chains with a central heme molecule, hemoglobin shows a spectrophotometric absorbance peak between 400 and 420 nm, which has been exploited for the quantification of hemoglobin in solution by Harboe and others [5,14,15]. As this characteristic can be used for hemoglobin quantification not only in solution but also when cell-bound, we have developed a spectrophotometric assay for assessing erythroid proliferation based on absorbance at 405 nm.

2. Materials and methods

All chemicals were obtained from Sigma–Aldrich (Arklow, Ireland) unless stated otherwise.

2.1. Cell isolation

Mononuclear cells (MNC) were isolated from peripheral blood buffy coats obtained from the Irish Blood Transfusion Services (Dublin, Ireland) using density gradient centrifugation with histopaque-1077. CD34+ cells were isolated from mononuclear cells via immuno-magnetic separation using anti-CD34 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bisley, UK).

2.2. Cell culture

Cultures were initiated from frozen or freshly isolated mononuclear cells or CD34+ cells. For depletion of leukocytes, mononuclear cells were pre-cultured for 2 days in IMDM with stabilized glutamine (Biochrom, Berlin, Germany) containing 5% (vol/vol) heparinized human plasma and 3 U/ml EPO (Janssen-Cilag, Dublin, Ireland). Cells were then washed and cultured in erythroid proliferation medium [12] consisting of IMDM with 330 μg/ml iron-saturated human transferrin and 10 μg/ml recombinant human insulin, supplemented with 5% heparinized human plasma, 100 ng/ml stem cell factor (SCF) (Cambridge Biosciences, Cambridge, UK), 5 ng/ml interleukin-3 (IL-3) (R&D Systems, Minneapolis, MN, USA), 3 U/ml EPO and 10^{-6} M hydrocortisone for 1–2 days. CD34+ cells were plated directly into the appropriate conditions. Manual cell counting was performed using the trypan blue exclusion method with trypan blue diluted 1/6 in phosphate buffered saline (PBS) and added to cells at 1:1 or 1:4 ratios.

2.3. Plasmodium falciparum *conditioned medium*

P. falciparum-conditioned medium was obtained from blood-stage cultures of *P. falciparum* 3D7 cultures grown in RPMI medium supplemented with 5% (wt/vol) Albumax® in a candle jar according to published methods [8]. *P. falciparum* cultures were grown to 10–15% parasitemia, washed two times with wash medium consisting of RPMI supplemented with 0.18 g/l sodium bicarbonate and one time with IMDM and then resuspended in IMDM. For higher protein content, cultures were concentrated 6–8 fold by resuspension in lower volumes of IMDM after washing and cultured for 3–4 h to obtain conditioned medium. Conditioned medium was obtained by centrifuging the culture supernatant for 10 min at 2000 rpm ($823 \times g$) followed by filtration through a 0.2 μm filter and used at up to 80% (vol/vol) in erythroid medium.

2.4. Absorbance-based assay

Cells (CD34+ cells or pre-cultured MNCs) were subsequently seeded in erythroid proliferation medium containing 5% heparinized human plasma, 100 ng/ml SCF, 5 ng/ml IL-3 and 3 U/ml EPO as standard conditions. These conditions served as a positive control for erythroid proliferation. As a negative control, cells were seeded in pure IMDM medium lacking growth factors and plasma. Erythropoiesis inhibiting agents were added at different concentrations or growth factor concentrations were varied to assess the effect on erythroid proliferation. Cells were seeded in 96-well flat-bottom plates at $1–5 \times 10^5$ cells/ml and cultured in a humidified incubator at 37 °C and 5% CO_2 for up to 21 days. All conditions were set up in triplicate and for each condition a well containing the appropriate medium blank without cells was included.

Absorbance measurements of plates with lid were taken at 405 nm using a Synergy H1 (Biotek, Potton, UK) plate reader preheated to 37 °C and following 2 min of linear shaking at 567 cycles per minute (cpm).

2.5. Analysis

Results from manual cell counting were determined as the mean and standard deviation of the cell concentrations of triplicate wells.

Results from spectrophotometric measurements were determined as the mean absorbance of triplicate wells and their standard deviation. The mean values were corrected by the absorbance of the medium blank and presented as percentage of the positive control. Statistical analysis was performed using Prism software (La Jolla, CA, USA).

3. Results

3.1. Method principle

The Harboe method has been established for determining hemoglobin concentrations in solution using spectrophotometric measurement at 415 nm and has been validated for assessing hemolysis in red cell samples [14,15]. We adapted this method for estimating erythroid cell concentrations in unlysed culture samples and determining erythroid proliferation in a non-invasive manner. Hemoglobin shows maximum light absorption between 400 and 420 nm and we found absorbance at 405 nm and 415 nm to correlate linearly ($R^2 = 0.9999$) allowing the use of 405 nm absorbance filters commonly available on standard plate readers (Fig. 1a). We established that the lysis of erythrocytes is not necessary for reliable hemoglobin quantification and that cell suspensions could be directly subjected to spectrophotometric measurement (Fig. 1b, $R^2 = 0.9905$). Initial assay set-up was performed using samples of native erythrocytes isolated from donor blood suspended in PBS and absorbance measurements at 405 nm were found to correlate linearly ($R^2 = 0.998$) with manual cell counts (Fig. 1c). Using the function obtained from the linear fit of such an erythrocyte standard curve using GraphPad software, cultures could be expressed as 'erythrocyte equivalents' based on