The cultured red blood cell: a study tool with therapeutic perspectives

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The erythroid line is certainly one of the best described of haematopoiesis and has been for a long time. The molecular mechanisms regulating its differentiation have formed the subject of numerous studies. Surprisingly, however, the enucleation which characterizes the final stage of maturation of the red blood cell (RBC) still remains very largely unexplained. What a paradox for a cell line of which the physiological role is major (transport of oxygen by haemoglobin), the pathology multidisciplinary (haemoglobinopathies, haemopathies, parasitic infections) and the therapeutic interest vital (blood transfusion).

One of the principal characteristics of the human RBC is in fact that it is the only cell to have a prolonged life span (120 days) despite the absence of a nucleus. The mechanisms of enucleation are suspected but have not been formally established due to a lack of experimental conditions permitting the massive *ex vivo* generation of RBC. Such conditions must indeed satisfy three requirements: the massive amplification of primitive haematopoietic stem cells (HSC), the controlled induction of exclusive differentiation to the erythroid line and the completion of terminal maturation to the stage of enucleated cells. If it is relatively easy to obtain selective erythroid differentiation, the data of the literature nevertheless show either strong cell proliferation without terminal maturation**,** or enucleation but with reduced cell amplification**.** No *ex vivo* system has yet been described which allows one to obtain both massive proliferation and enucleation of all the erythroblasts.

We have previously reported (*Nature biotechnology, 2002; 5; 464-472*) a protocol for the expansion of HSC derived from cord blood (CB) in a well defined medium without stroma, based on the sequential addition of growth factors. Starting from $CD34^+$ cells, this protocol permits the massive production (up to 200,000 fold amplification) of pure erythroid precursors (95 to 99 %). Contrary to our observations *ex vivo*, when these progenitors/precursors are injected into non-obese diabetic severe combined immunodeficient (NOD/SCID) mice, they are capable of continuing to proliferate *in vivo* and of differentiating in four days to the terminal stage of enucleated cells, which confirms the major role of the microenvironment in terminal erythroid differentiation.

In adult humans *in vivo* haematopoiesis is in fact obtained through a dynamic production process taking place in the bone marrow, which starts from a minor population of HSC and proceeds according to a pyramidal cell hierarchy (stem cells, progenitors and mature cells), in close contact with the microenvironment. The stromal cells play a decisive role in the secretion of soluble regulatory factors and as cells of the extracellular matrix. Intercellular contacts and soluble activators and inhibitors are the key elements of the regulation of haematopoiesis. On the basis of these facts, we have designed a new protocol for the expansion and differentiation of $CD34^+$ cells from blood, marrow or CB in three steps (*Nature Biotechnology, 2005; 23,1; 69-74*): a first phase (in liquid medium) of proliferation and

induction of erythroid differentiation in the presence of stem cell factor, interleukin-3 and erythropoietin (Epo), a second using a model reconstitution of the microenvironment (murine stromal line MS5 or human mesenchymal cells) in the presence of Epo alone and a third phase in the presence of the stromal cells alone, without any growth factors.

Massive amplification of erythroid cells

This protocol allows both the massive expansion of $CD34^+$ stem cells/progenitors and their complete differentiation to the stage of mature, perfectly functional RBC. Thus, on day 15 the plateau of cell amplification is of the order of 15,000 fold for $CD34^+$ cells derived from bone marrow or peripheral blood, 30,000 fold for those obtained by cytapheresis after mobilization with the granulocyte growth factor G-CSF and 140,000 fold for those derived from cord blood. On day 15, 98 % of the cells are reticulocytes. By day 18, almost 100 % of the cells are enucleated RBC having all the morphologic characteristics of native RBC. The proliferation can be increased to $2x10^6$ fold simply by prolonging the first phase for three additional days.

The cultured reticulocytes and RBC are perfectly normal with regard to: (1) their enzymatic content (glucose-6-phosphate dehydrogenase, pyruvate kinase), as shown by their ability to reduce glutathione and maintain the concentration of ATP (to avoid the accumulation of 2-3 diphosphoglycerate which would decrease the affinity of haemoglobin); (2) their membrane, which ensures a perfect deformation capacity (evaluated by ektacytometry and protein analysis); (3) their haemoglobin which behaves like tetrameric haemoglobin, capable of binding and releasing oxygen (established by laser flash photodissociation and measurement of the oxygen saturation); (4) their ability to survive *in vivo*, assessed by injection into NOD/SCID mice.

A multidisciplinary field of application

Study of haemoglobin (Hb): The nature of the Hb synthesized in these cultured RBC depends on both the origin of the $CD34^+$ cells and the culture conditions. In fact, the RBC obtained from $CD34^+$ cells derived from bone marrow or peripheral blood contain 95 % HbA, whereas those obtained from cord blood $CD34^+$ cells contain predominantly fœtal Hb (HbF) (64 %). This *ex vivo* synthesis of HbF in cells generated from cord blood is undoubtedly linked to the culture conditions, since the erythroblastic precursors obtained after 10 days of culture in the absence of a microenvironment give rise *in vivo,* after injection into NOD/SCID mice, to mature RBC containing 96 % functional HbA. Hence this model constitutes a tool to study the cellular and molecular mechanisms of haemoglobin synthesis. Insofar as one can partly modify the Hb expression profile by using different culture conditions, one may hypothesize that *in vitro* stimulation of the synthesis of HbF in the progenitors of patients suffering from severe haemoglobinopathy (drepanocytosis, thalassemia) might represent an *ex vivo* therapeutic approach.

Myelodysplastic syndromes: These constitute a malignant pathology in constant progression. Primarily the erythroid line is affected and the medullar insufficiency is characterized notably by an absence of

terminal maturation of the erythroid precursors. Numerous physiopathological hypotheses have been advanced, implicating in particular the microenvironment. The cultured RBC model provides a new tool to study in parallel the erythroid compartment and the microenvironment in these pathologies.

Blood transfusion: RBC culture technology is potentially applicable to blood transfusion. In fact, the quantities which one should be able to produce are compatible with clinical requirements (a standard red blood cell concentrate contains about $2x10^{12}$ RBC). If one considers that one cord blood donation contains 2 to $5x10^{6}$ $CD34^{+}$ cells or that cytapheresis mobilized with G-CSF permits the recovery of 4 to $8x10^{6}$ $CD34^{+}$ cells per Kg of body weight and that the levels of amplification are respectively of the order of $2x10^{6}$ and 10^{5} fold, it is certainly the equivalent of several RBC concentrates which can be produced from a single donation. Over and above the obvious interest for transfusion in terms of supply and infection safety (several units produced from a single donor and/or autologous transfusion patient), this type of product is also promising from the point of view of transfusion efficacy. It would enable the transfusion of a cell population homogeneous in age of which the life span should be close to 120 days, as compared to the mean half-life of 28 days of the RBC collected from a donor, a consequence of the simultaneous presence of RBC differing in age. This would reduce the number of transfusions and alleviate the inevitable iron overload, a major complication in polytransfused patients. The adaptation of the model to a clinical application implies the establishment of clinical grade production conditions, notably through preferential use of stromal cells of human origin.

Parasitology: This model of erythroid differentiation may constitute a simple new tool for study of the reproduction cycle of certain infectious agents like the malarial parasite. Until now immunodeficient mice represented the only model allowing study of the plasmodium with the aim of developing new drugs or optimizing antimalarial vaccines. The limited life span of human RBC injected into SCID mice further necessitated a complex preparation of the model recipient (splenectomy, irradiation, drug treatment …). Other groups have developed methods of isolating reticulocytes from the mice or from patients presenting haematological pathologies. The cultured RBC, easily accessible, circumvents the complexity of *in vivo* modelling.

A new drug vector: RBC have by nature an ideal biodistribution and no longer divide. Such properties could be exploited to use these cells as a new type of therapeutic vector. Thus, prior to the induction of erythroid differentiation, the progenitors could be genetically manipulated to produce cytoplasmic or membrane proteins having a deliberately limited duration of action, for a specific therapeutic objective.

Perspectives: Human embryonic stem cells (huESC), which have an unlimited proliferative potential, could become in the next few years an alternative and attractive source for cell engineering. Some groups have already reported a 20% commitment of huESC to $CD34^{+}$ haematopoietic cells. Hence *in vitro* differentiation of ESC to the erythroid line could eventually permit the massive production of RBC.

Conclusion

This new concept of “cultured RBC” provides an innovative tool to elucidate the physiopathological mechanisms of erythropoiesis and opens up potentially considerable therapeutic perspectives, notably in the field of blood transfusion. The hopes engendered by this new approach to cell therapy should become concrete reality in the relatively near future, given the right conditions of Research and Development.