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NEOCYTOLYSIS OF RED BLOOD CELLS FOLLOWING HIGH ALTITUDE EXPOSURE

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Abstract

Introduction: The cellular changes, driven by hypoxia exposure, represent an adequate response to the high altitude environment making newly generated RBCs more fit to manage oxygen uptake and delivery.

Objective: To answer the following questions:

1) Are these changes still advantageous upon return to normoxia?

2) Does HIF mediated EPO's increase, in high altitude give rise to a plethora of scarcely selected, hypoxia adapted neocytes whose phenotype makes them susceptible to phagocytosis, upon return to normoxia?

3) Does EPO's decrease, occurring during de-acclimatisation, the main cause triggering neocytolysis, as the abrogation of neocytolysis *in vivo* by EPO administration seems to suggest?

Methods: A first group of 4 mountain climbers was studied (age 28–43 years, 2 males and 2 females). Their hematological parameters and RBC phenotype were analysed before and after 53 days acclimatization at high altitude (≥ 4500 m). The RBCs populations were fractionated by density separation into age-based subsets (young, middle-aged and old), the RBCs counts of the three age classes were assessed and some phenotypical features of RBCs from these subsets were investigated by flow cytometry. In particular, the expression of CD55 and CD59 that are partially lost by RBCs during in vitro and in vivo aging were measured. The expression of CD47 and phosphatidylserine (PS) exposure on the outer membrane were also measured. In a second group of five mountain climbers (age 41-53 years, 4 male, 1 female,) possible changes in the expression of fetal haemoglobin was analysed by flow cytometry, western blotting and quantitative RT-PCR performed on reticulocytes and neocytes generated at high altitude (17 days at 3100 -5600 m a.s.l.).

Results: Upon return to sea level a dramatic decrease in the number of RBCs in the low and middle density subsets and a corresponding increase of cells in the dense subset was observed. Moreover, the analyses showed a shift to a "senescent-like" phenotype in all RBCs subpopulations as shown by the decrease of expression of CD55, CD59 and CD47 and by increased exposure of PS. EPO concentration was lower in all subjects as compared with the values measured in control blood samples. In the second group of mountain climbers, a slight but significant Increase In fetal hemoglobin expression by neocytes generated at high altitude was observed (from 1% to 12% of circulating RBCs).

Conclusions: It is likely that the changes induced by high altitude, such as the increased expression of fetal hemoglobin, make the red cells function more effective even at low PO_2. targeting possibly the neocytes to phagocytosis once the mountain climbers return to sea level, i.e.to normoxia. Beside the membrane changes the presence of fetal haemoglobin could imbalance the oxygen uptake/delivery by RBCs in normoxia making them more susceptible to oxidative stress and, ultimately, directing them to "senescence" and phagocytosis.

Key words: *hypoxia, high altitude, neocytolysis, erythropoietin, HIF*

Introduction

The discovery of erythropoietin (EPO) and of its key role in erythropoiesis allowed a comprehension of the erythropoietic response to hypoxia in molecular terms. Furthermore, studies on the activation of EPO gene paved the way for the identification of the oxygen sensing HIF (Hypoxia Transcription Factor) pathway controlling a wide range of tissue and system specific responses to hypoxia. Genomic analysis of the Tibetan highlanders has revealed a selection that has favoured variants of HIF-2 alpha associated with a haematological profile similar to that of Tibetan lowlanders (i.e.: concentration of haemoglobin and haematocrit within normal ranges). On the basis of these data, it has been suggested that the HIF-mediated increase of erythropoiesis is a misdirect response to hypobaric hypoxia that originally evolved as a response to anemia. In keeping with this conclusion is the process occurring upon transition of lowlanders from hypoxia to normoxia (for instance, mountain climbers returning to sea level after high altitude acclimatization), which consists of a decrease of EPO plasma level and a fast reduction of erythrocyte mass through neocytolysis, i.e. lysis of young erythrocytes.

Neocytolysis has been observed in healthy subjects after return from high altitude to sea level and is involved also in anemias associated to reduced production of EPO, for instance in renal diseases.

In subjects exposed to conditions triggering neocytolysis, beside the dramatic reduction of young RBCs counts, changes in neocytes membrane components have been observed contributing to a "senescent-like"

phenotype and likely targeting them to macrophage phagocytosis.

Of the several cells and tissue types sensitive to hypoxia, red cells and the erythropoietic system have been one of the most extensively studied for years both in man and other mammals.

Over 100 years ago the association between hypoxia and increase of erythrocyte mass was demonstrated [1,2]. The discovery of erythropoietin (EPO) and of its key role in erythropoiesis allowed a comprehension of this process in molecular terms. Furthermore, studies on the activation of EPO gene paved the way for the identification of the molecular machinery that controls a wide range of tissue specific and systemic responses to hypoxia. Researchers focused mainly on genetic and functional features of the proteins which are components of HIF (Hypoxia Transcription Factor). This factor, an heterodimer including one of the three subunit alpha 1, 2, 3 and a beta subunit, activates the transcription of genes coding for proteins mediating the adaptive response to hypoxia, such as erythropoetin and some enzymes of the glycolytic pathway [3,4].

Recent genomic analysis of the Tibetan highlander population, that has been living at high altitude since 25000 years, has revealed that HIF-2 alpha, the oxygen sensitive subunit involved in the regulation of erythropoiesis, and genes related to the HIF signalling pathway, have been subjected to strong and recent positive selection [5,6]. In particular, a positive selection has favoured variants of HIF-2 alpha associated with reduced blood concentration of hemoglobin and reduced haematocrit. This is associated with the haematological profile of Tibetan highlanders which is similar to the one typical of lowlanders. Indeed, an increased haemoglobin concentration and haematocrit, while can be advantageous under hypoxia, results in an elevated blood viscosity, thereby compromising tissue oxygenation and ultimately survival at high altitude.

On the basis of these data, it has been suggested that the HIF-mediated increase of erythropoiesis is a misdirect response to hypobaric hypoxia that originally evolved as a response to anemia [7].

In keeping with this conclusion is the process occurring upon transition of lowlanders from hypoxia to normoxia (for instance, mountain climbers returning to sea level after high altitude acclimatization), which consists of a decrease of EPO plasma level and a fast reduction of erythrocyte mass through neocytolysis, i.e. lysis of young erythrocytes.

Neocytolysis has been observed in healthy subjects after return from high altitude to sea level and has been shown to occur also in astronauts [8-10]. In fact in microgravity, a central blood poling occurs, associated extracellular fluid release into tissues. The consequent increased diuresis and decreased plasma volume cause haematocrit augmentation, leading to EPO decrease and neocytolysis, so that red cells mass is reduced in a few days [9].

Neocytolysis is involved also in anemias associated to reduced production of EPO, for instance in renal diseases [11]. It may be caused also by artificially induced EPO fluctuations, in cases of blood doping.

In subjects exposed to conditions triggering neocytolysis, beside the dramatic reduction of young red cells counts, changes in neocytes membrane components have been observed, and that contributes to a "senescent-like" phenotype and likely targets them to macrophage phagocytosis [10]. The mechanism(s) leading to these membrane changes are not known, nor it is known if, and how, they could be related to decreases in the level of EPO, or other factors, in plasma. Evidence from the literature seems to support both views. After hypoxia exposure, neocytolysis could eliminate those red cells, that, after a maturation from erythroid precursors under low oxygen partial pressure condition, could have phenotypic and functional properties not appropriate to a normoxic environment. Indeed a different composition in membrane lipids [12], a higher concentration of intracellular ATP (10), and a partial fragmentation of actin [13], have been observed in a fraction of red cells populations from mountain climbers after high altitude acclimatization and return to sea level. More recently, the generation of RBCs containing fetal hemoglobin driven by hypoxia exposure has been shown [14].

Investigations about the "precarious" life of young erythrocytes, in conditions of fluctuating erythropoietin levels, as a result of physiological adaptation to extreme environments or of nephropathy, are presently conducted. The methods used for the separation of neocytes and the analysis of their biochemical and functional features have been described in several scientific reports and appropriately changed by the researcher author of this proposal. To obtain age-ranked erythrocyte populations from blood samples, red cells have been separated into three density subsets (low, middle and high density) by centrifugation on discontinuous density gradients of Percoll® [10]. It is known that red cells density increases with age.

The neocytes separated from the whole RBCs population have been subjected to a number of analysis performed at cellular and molecular level. These studies tried to answer to the following questions:

1) Do the cellular changes, driven by hypoxia exposure, represent an adequate response to the high altitude environment making newly generated RBCs more fit to manage oxygen uptake and delivery? But are these changes still advantageous upon return to normoxia?
2) Does HIF mediated EPO's increase, in high altitude give rise to a plethora of scarcely selected, hypoxia adapted neocytes whose phenotype makes them

susceptible to phagocytosis, upon return to normoxia?

3) And does EPO's decrease, occurring during deacclimatisation, the main cause triggering neocytolysis, as the abrogation of neocytolysis *in vivo* by EPO administration seems to suggest [8]?

Materials and methods

To deal with these issues we studied a group of four mountain climbers (age 28–43 years, two males and two females). Their hematological parameters and RBC phenotype were analysed before and after 53 days acclimatization at high altitude (≥ 4500 m). The RBCs populations were fractionated by density separation into age-based subsets (young, middle-aged and old), the RBCs counts of the three age classes were assessed and some phenotypical features of RBCs from these subsets were investigated by flow cytometry. In particular, the expression of CD55 and CD59 [15] that are partially lost by RBCs during *in vitro* and *in vivo* aging [16] were measured. The expression of CD47 [15] and phosphatidylserine (PS) exposure on the outer membrane were also measured. These molecules seem to be involved in the negative [17-19] and positive [20-21] regulation, respectively, of red cell phagocytosis.

Results and discussion

Upon return to sea level, this analysis showed a shift to a "senescent-like" phenotype in all RBCs subpopulations, characterised by a decrease in the level of expression of CD47 in young and middle-aged RBC subsets, exposure of PS on the membrane outer layer and a decline in the expression fluorescence intensity of CD55+ and CD59+ as measured by flow cytometry.

EPO concentration was lower in all subjects as compared with the values measured in control blood samples (mean values: 2.5±3.3 vs. 10 ± 4.5 mIU/ml, $P < 0.05$).

Finally, after the 6-day descent to sea level, we observed a dramatic decrease in the number of RBCs in the low and middle density subsets and a corresponding increase (more than 3 times the control levels) of cells in the dense subset.

Further analysis of proteomes was undertaken to investigate possible changes in the membrane skeleton of these "hypoxia" adapted RBCs. By two-dimensional electrophoresis and by mass spectrometry we analysed the proteins of red cells ghosts obtained from our subjects before and after return to sea level. After the hypoxia exposure, we observed a lower expression (- 73%) and fragmentation of the same molecule (42 kDa) in 2 fragments of 27 and 28 kDa respectively [13]. This suggested an alteration in membrane skeleton structure, which was confirmed by beta-actin release in cells lysates during the ghost preparation. We observed a similar actin fragmentation and release in red cells lysates from beta-thalassaemic patients. Then, after hypoxia exposure, RBCs displayed actin modification and cytoskeleton instability.

Finally, data from a study we performed on a different group of mountain climbers who lived at high altitude (ranging from 3100 to 5600 m above sea level, Nevado Copa mountain in Peru) for 17 days, strongly support the hypothesis that, like in other instances of stress erythropoiesis such as in thalassemia or sickle cell syndromes, also in hypoxia more RBCs containing fetal hemoglobin, named fetal RBCs, are generated (in our study 5.3 times more than in normoxia). This was evaluated by comparative flow cytometry analysis of RBCs drawn before, during and after the high altitude dwelling, and the data were also supported by western blotting of partially purified hemoglobin and hemolysates of neocytes and by Q-RTPCR of m-RNA of reticulocytes enriched fractions from blood samples drawn at the three time points above mentioned [14].

The latter observation is in agreement with the notion of an hypoxia adaptation in red cells, which could shape an RBCs phenotype affecting either membrane molecules and haemoglobin The changes make the red cells function more effective even at low PO_2 targeting possibly the neocytes to phagocytosis once the mountain climbers return to sea level, i.e. to normoxia. In fact, beside the membrane changes the presence of fetal haemoglobin could imbalance the oxygen uptake/delivery by RBCs in normoxia making them more susceptible to oxidative stress and, ultimately, directing them to "senescence" and phagocytosis.

Further studies are now focused on the identification of the role of EPO or other possible factors in the survival of neocytes cultured in vitro in the presence of autologous or fetal calf serum. Survival of whole red cells populations in vitro incubated, is prolonged by autologous plasma and the protection is mediated by Bcl-X(L) [22]. Studying neocytolysis in a number of physiological and pathological systems may be beneficial in the treatment of some particular blood diseases, and furthermore it may be also helpful to pave the way for the biochemical identification of hypothetical "survival factors" acting on RBCs. Moreover, studying the factor(s) that tip the balance toward survival or destruction in a relatively simple system such as the red cell can provide a model to understand maturation and survival of other cell types in other tissues. Indeed, EPO acts also on endothelial cells and on cells of the central nervous system [glia cells and neurons, ref. 23].

Conclusions

Beside the pay-load for basic science, some benefits can come from this study also for the treatment of the anaemia of uraemic patients unresponsive, or inappropriately sensitive, to EPO.

Finally, results from the proposed study may find potential application in the analysis to detect EPO abuse by athletes who want to enhance their performance. In case of EPO doping neocytolysis has not been studied, but the prediction of the theory is that, since this process is strictly related to EPO fluctuation, neocytes of athletes would be doomed to destruction after EPO withdrawal. The possibility to reveal an ongoing neocytolytic process, by analysing some property of the circulating neocytes, could be a useful complement to the commonly used analysis of urine. Although this latter method is very sensitive and reliable, it is unpractical for many laboratories and its application is limited to the detection of EPO shortly after its injection, or within a short period of time (2-3 days) since the last administration of the hormone. Neocytolysis takes 5-7 days, as shown by the group of Alfrey and Rice [8] and as reconfirmed by Risso et al. (unpublished data). Then the precise identification of neocytes, their biochemical characterisation and the definition of a "senescent" phenotype that favour cell destruction could be useful, especially when the urine analysis cannot be performed.

Declaration of interest

The authors report no conflicts of interest.

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