Increased hypoxic proliferative response and gene expression in erythroid progenitor cells of Andean highlanders with chronic mountain sickness

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Submitted 19 August 2019; accepted in final form 10 October 2019

INTRODUCTION

The excessive production of red blood cells (RBCs) or excessive erythrocytosis (EE) is considered an indicator of poor adaptation to life at high altitude (6, 35, 37, 43, 45). EE is the main sign of a clinical syndrome known as chronic mountain sickness (CMS) or Monge’s disease, common to Andean and other high-altitude populations around the world (13, 36, 44, 57). Hypoxemia and neurological symptoms such as headache, fatigue, and alterations of sleep and memory usually accompany EE (3, 26, 30, 33, 49, 53). Pulmonary hypertension (38, 40) and cerebral, cardiovascular, and pulmonary accidents caused by blood hyperviscosity and thromboembolic events (2, 25, 26, 2) are also frequent in CMS patients. The prevalence of CMS increases with altitude, and the condition develops insidiously from early adulthood, progressing with age (27, 34, 36). Symptoms can significantly affect the quality of life in native highlanders, as the condition frequently becomes incapacitating. Several studies have shown that the common practice of hemodilution at the altitude of residence alleviates CMS-related symptoms (11, 24, 54, 56), indicating that symptomatology is secondary to EE. The pathophysiological mechanism that results in the occurrence of EE, however, is still controversial, although systemic physiological alterations in terms of respiratory, cardiovascular, and hormonal responses to chronic hypoxemia have been proposed as potential causes to explain the exacerbated erythropoietic response (4, 18, 29, 47). These alterations might be associated with differential polymorphisms in genes related to the regulation of erythropoiesis, angiogenesis, cardiovascular, and steroid-hormone function, among others, identified by whole-genome studies in CMS and non-CMS highlanders (4). In particular, a number of studies suggest that sentrin-specific protease 1 (SENP1), which regulates the activity of transcription factors such as hypoxia-inducible factor (HIF) and GATA-binding factor (GATA), has a central role in the excessive production of RBCs, possibly by modulating different stages of erythro-
poiesis, including steps of the erythropoietin (EPO) signaling pathway and apoptosis in erythroid progenitors (5, 8, 58).

EPO is an essential hypoxia-responsive erythropoietic hormone that mediates the survival, proliferation, and differentiation of erythroid progenitors (17, 23). Under hypoxic conditions, EPO levels increase, stimulating the production of RBCs and elevating hematocrit to a new steady state (32). However, among Andean highlanders, most studies show that despite severe hypoxemia, serum EPO concentrations in CMS and non-CMS individuals are similar, although the former show significantly higher hematocrit values. Elevated serum EPO accompanies only extreme hematocrit values (12, 28). We have previously shown that decreased plasma concentration of the soluble EPO receptor (sEPO), an endogenous EPO antagonist, is among the factors that might explain EE despite altitude-normal serum EPO in CMS highlanders, because increased blood EPO availability to bind membrane EPOR would result in a stronger erythropoietic stimulus (50, 51).

Another suggested mechanism includes increased local expression of EPO in the bone marrow, associated with increased hypoxia inducible factor α levels (48). However, erythroid progenitor cells of CMS individuals could, hypothetically, be more sensitive to EPO, and hence their proliferative response to hypoxia would be stronger than in non-CMS cells.

Under in vitro hypoxic conditions, CMS erythroid progenitors derived from human induced pluripotent stem cells (iPSCs) obtained from skin fibroblasts show increased proliferation and increased SENP1 expression, associated with stabilization and upregulation of GATA-binding factor 1 (GATA1) and GATA1-responsive genes such as the mitochondrial anti-apoptotic factor Bcl-xL (5). These findings suggest a crucial role for SENP1 in erythroid proliferation seen in CMS. Still, whether physiological responses of iPSCs-derived CMS erythroid progenitors are the same as in native CMS cells needs confirmation. Besides, since the expression of Bcl-xL requires the activation of EPOR, also a GATA1 target (16, 58, 59), increased EPO sensitivity in erythroid progenitors is possibly associated with upregulated EPOR expression. Therefore, the aim of the present study was to determine the hypoxic proliferative response of native erythroid progenitors (burst-forming units-erythroid [BFU-E]) derived from CMS and non-CMS peripheral blood mononuclear cells (PBMCs); to examine the expression of SENP1, GATA1, EPOR, and EPO in BFU-E cell cultures; and also to investigate the functional upstream role of SENP1 in native erythroid progenitor differentiation into erythroid precursors in the absence of the influence of systemic factors.

MATERIALS AND METHODS

Ethical approval. The study was approved by the Institutional Ethics Committee of Universidad Peruana Cayetano Heredia and by the University of California San Diego, Human Research Protection Program. All participants signed an informed consent form in Spanish.

Study participants. Thirty-six participants, 17 CMS and 19 non-CMS native highlanders, were included in the study. A minimum sample size of 16 participants per group for BFU-E colony proliferation rate and total colony area as primary outcome variables was estimated using a Cohen’s d effect size value of 1 with a statistical power of 80% at P < 0.05. All participants were men, high-altitude natives, and lifelong residents of Cerro de Pasco, Peru (4,380 m), 20 to 65 yr old, and had at least two previous known generations of high-altitude (>3,000 m) Andean ancestry. Participants were excluded if they had history of pulmonary, cardiovascular, or renal disease; were current smokers; were miners; had undergone blood transfusions or phlebotomies in the previous six months; had traveled to lower altitudes (<3,000 m) for more than seven days during the previous six months; or had demonstrated abnormal cardiac or pulmonary function during screening procedures.

Preliminary screening, hematocrit, and CMS score. Clinical examination was performed during a preliminary screening session to rule out prior history of cardiovascular or pulmonary disease. During this session, pulse oxygen saturation (SpO2), heart rate, and systolic and diastolic blood pressure were measured. Hematocrit was determined from duplicate micro-centrifuged blood samples obtained from a fingertip capillary blood draw. Participants with hematocrit ≥ 63% (Hb concentration ≥ 21g/dl) were classified as individuals with EE (26). General health and CMS score questionnaires were also applied. CMS score determines the absence or presence and severity of the syndrome and is based on the occurrence of EE, as well as the following signs and symptoms: headache, shortness of breath or palpitations, sleep disturbances, paresthesia, cyanosis, dilated veins, and tinnitus (26). All participants were also interviewed about their history of high-altitude residence and ancestral background (self-identified ancestry and geographical location of their parents and grandparents).

Samples. Blood samples for PBMC isolation were obtained in three 10-ml sodium heparin-coated tubes. Two additional 6-ml blood samples were obtained in clot-activator-coated tubes to determine EPO and iron profile. Samples were taken between 5 AM and 7 AM to avoid variation in serum EPO due to circadian rhythm. Serum was separated by centrifugation and stored in liquid N2 until analysis.

Serum EPO and iron homeostasis indexes. A specific sandwich ELISA kit was used for serum EPO determination as described by the manufacturer (DRG International, Springfield Township, NJ). Each sample was run in duplicate. Iron, ferritin, and transferrin were measured in serum samples (Medlab clinical laboratories, ISO 9001:2000, Lima, Peru).

PBMCs isolation and cell culture. PBMCs were isolated using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) by gradient centrifugation. PBMCs were then cultured in Methocult H-4534 medium (StemCell Technologies, Vancouver, Canada) at a final density of 2.5 × 105 cells/ml in the presence of 3 U/ml of rhEPO (StemCell Technologies) for 14 days. Cell cultures were maintained at 37°C with 5% CO2 and 10% O2 for normoxic conditions in a Biospherix X3 hood (Biospherix, Parish, NY). Cellular normoxia for PBMCs was calculated as the fractional equivalent of the mean between average arterial and venous PO2 (PAO2 = 95 mmHg and PV(O2) = 45 mmHg, respectively) near sea level (barometric pressure [BP] = 747 mmHg), taking into account water vapor pressure (P, H2O) at 37°C. Therefore: [(PAO2 + PV(O2))/2]/(BP - P, H2O)] = 0.099 or 10%. For incubation under hypoxic conditions, O2 levels were lowered down to 1%.

BFU-E colony identification and count. Colony identification and count were performed at ×20 magnification on days 7 and 10 using an inverted LS 560 microscope (Etaluma, Carlsbad, CA) inside the hypoxic hood. On day 14, cultures were removed from the hood and analyzed using an AxiosZoom Stereomicroscope (Carl Zeiss Microscopy, Jena, Germany) at ×10 magnification. Digital images were analyzed with Zeiss ZEN software to calculate the area occupied by each colony. The rate of change in the number of colonies, or colony proliferation, was calculated as the slope of the relationship between number of colonies and days of incubation (from day 7 to day 14). No colonies were present at day 0. Total colony size was calculated as the product of the number of individual colonies by their area on day 14.

Real-time PCR. Expression of EPO and EPOR mRNA was determined in BFU-E colony cells by reverse transcription quantitative PCR (RT-qPCR), using 18S rRNA as the reference gene. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) and an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was generated using 500 ng of total RNA using Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA). Quantitative PCR was per-
formed in 10-μl reactions using LightCycler 480 SYBR Green I Master (Roche Life Science, Indianapolis, IN) and 100 pm of primers directed to EPO, EPOR mRNA, and 18S rRNA. PCR reactions were run in triplicate for each sample using 40 cycles of 2 min at 95°C and 1 min at 60°C on the PikoReal 96 platform (Thermo Fisher Scientific, Waltham, MA). SENP1 and GATA1 cDNA was produced from total RNA through reverse transcription-PCR using a Superscript Vilo IV (Applied Biosystems). Results were calculated as fold change and also as relative gene expression. Fold change was calculated to compare the expression levels of the target genes between the CMS and non-CMS groups using the Livak method \((2^{-\Delta\Delta Ct})\) taking the average of Δ threshold cycle (Ct) values of the non-CMS group at 10% O₂ as control group, \(2^{-\Delta\Delta Ct} = \text{CMS} - \text{ΔΔCMS}\). The relative expression ratio was used to compare the expression levels between each one of the target genes and the housekeeping gene, \(2^{\Delta Ct}\text{target} / 2^{\Delta Ct}\text{reference}\). Considering that the Ct of the reference gene is the same for both the CMS and non-CMS groups at both O₂ concentrations, then the ΔΔCt equals 0 and the former equation can be simplified to \(2^{\Delta Ct}\text{target}\) (38).

Generation of erythroid precursor (CD235a cells) from native PBMC-derived erythroid progenitor (CD34+) cells of CMS and non-CMS subjects under normoxia and hypoxia. Erythroid precursors (CD235a cells) were generated from PBMCs by gradient centrifugation using Histopaque. Dynabeads CD34+ Isolation Kit (Invitrogen, CA) was used to purify the CD34+ fraction. CD34+ cells were expanded for a week (days 0–7) in Stem span medium containing Hydrocortisone (10-3 M), SCF (50 ng/ml), FLT3L (50 ng/ml), IL3 (10 ng/ml), BMP4 (1 ng/ml), IL-11 (40 ng/ml), and EPO (2 U/ml). After expansion, cells were further differentiated using the protocol from Giarratana et al. (17a). Cells were then cultured in erythroid differentiation medium (EDM) on the basis of IMDM supplemented with stabilized glutamine, 330 μg/ml holo-human transferrin, 10 μg/ml recombinant human insulin, 2 IU/ml heparin, and 5% plasma. In the second step (days 7 to 11), cells were resuspended at 10^5/ml in EDM supplemented with SCF and EPO. In the third step (days 11 to 18), cells are cultured in EDM supplemented with EPO alone. Cell counts were adjusted to 7.5 × 10^5 to 1 × 10^6 and 5–10 x 10^5 cells/ml on days 11 and 15, respectively. Beyond day 18, the culture medium containing EPO was renewed twice a week and cultures were maintained at 37°C in 5% CO₂ under normoxic or hypoxic conditions; results are presented in terms of the actual rate of expansion after plating. For flow cytometric analysis, erythroid bodies were dissociated using Accutase Cell Disassociation reagent (Invitrogen, Carlsbad, CA), washed with PBS supplemented with 2% FBS, and filtered through a 70-μm cell strainer (Falcon; BD). Cells were treated with propidium iodide (Sigma-Aldrich) before analysis. Cells were hCD235a-PE (glycophorin A) from BD and analyzed by a FACSCanto cell analyzer (BD) using FACSDiva software (version 6.0; BD).

SENP1 knock-down. For SENP1 knock-down, packaging and lentivirus generation was done by Salk Institute Gene Transfer, Targeting, and Therapeutics Core. Transduced cells were selected at 0.5 μg/ml puromycin (Sigma-Aldrich). The cells were transduced at CD34+ cell stage and selected by puromycin selection before expansion as discussed in the protocol above.

Statistical analysis. STATA 15 software was employed for statistical analysis. Normality of distribution and homogeneity of variance were assessed for comparison between continuous variables. Student’s t test for equal variances was applied as parametric test and Wilcoxon t test as nonparametric test to evaluate differences between CMS and non-CMS cell culture outcomes and to compare expression levels of EPO and EPOR in CMS and non-CMS cells cultured under hypoxia and normoxia, whereas Student’s t-test was performed to compare the expression of SENP1 and GATA1. In general, values of \(P < 0.05\) were considered statistically significant.
Figure 3 shows representative FACS analysis images of native erythroid cells. FACS analysis showed that in normoxia, CMS and non-CMS CD34+/H11001 cells generate similar relative proportions of erythroid precursors (CD235a cells, Fig. 3A, i and ii, and Fig. 3B). However, under hypoxic conditions, CMS CD34+ cells generated a significantly larger proportion of CD235a cells (Fig. 3A, iii and iv, and Fig. 3B). Figure 3C shows that SENP1 knock-down in CMS cells reduced the relative proportion of CD235a to similar levels found in non-CMS cells.

DISCUSSION

The excessive hematological response observed in a significant part of the Andean population who suffer from EE and CMS is undoubtedly an indicator of maladaptation to life at high altitude. We generated native erythroid progenitor cells from CMS and non-CMS individuals to search for core cellular mechanisms in the absence of systemic factors. Our main finding shows an intrinsic exacerbated erythropoietic response to hypoxia in erythroid progenitor CMS cells together with increased gene expression of SENP1, GATA1, and EPOR, with SENP1 as a crucial upstream regulator.

The role of serum EPO has been controversial concerning the development of EE in Andeans. Typically, serum EPO values are similar in CMS and non-CMS highlanders, and there is no correlation with hematocrit or hemoglobin concentration (18, 22, 28, 48, 51). Only in few cases, CMS individuals show elevated EPO, which usually associates with extreme hematocrits (12, 28, 51), and therefore it is unlikely that this sole factor explains the excessive RBC production. Recently, we have shown that plasma sEPOR concentration and the EPO-to-sEPOR ratio, as an index of EPO availability, are better systemic predictors of hematocrit in CMS than EPO alone (50). Here, we confirm similar serum EPO values in CMS and non-CMS highlanders, and their lack of association with EE.

We also demonstrate that under similar EPO concentration in vitro, CMS cells show a stronger proliferative response, reflected in the higher proliferation rate and larger total colony size during culture under hypoxic conditions, compared with non-CMS cells. Interestingly, the latter did not show a significant increase in either proliferation rate or total colony size, which suggests a relatively blunted erythropoietic response. A similar but more pronounced blunting was reported in hypoxic PBMCs-derived BFU-E colonies obtained from Tibetan natives, typically known for lower hematocrits compared with Andeans at similar altitudes (31). These cells showed decreased EPO sensitivity and significantly less proliferation and colony size compared with colonies from control lowlanders.
The study showed that this reduced erythropoietic response was related to missense variants of the \( EGLN1 \) gene. These variants are associated with the relatively low Hb values observed in Tibetans and are considered protective alleles against high-altitude polycythemia (46). However, we have recently shown that these \( EGLN1 \) variants are absent or in very low frequency in Andeans of this same population of Cerro de Pasco (21), and therefore the partially blunted erythropoietic response seen in Andean non-CMS BFU-E cells is most possibly related to different alleles or epigenetic modifications (10, 14, 15).

A key point in our study is the increased expression of \( SECP1 \) and \( GATA1 \) in CMS erythroid progenitors, confirming the findings on iPSC-derived CMS erythroid cells. Under hypoxic conditions at 5% O\(_2\), iPSC-derived CMS erythroid cells display a robust proliferative response to form RBCs (5). Also, the study showed that \( SECP1 \) plays a critical role in the differential erythropoietic response of CMS versus non-CMS cells. The results strongly suggest that \( GATA1 \) is a down-stream \( SECP1 \) target and has a critical antiapoptotic effect in these cells. \( GATA1 \) drives the expression of many erythropoietic and antiapoptotic genes, and it is also required for EPOR expression (9, 60). However, the study did not find a differential expression of EPOR between CMS and non-CMS cells. In our study, we observed a modest increase in EPOR fold- and relative expression in CMS compared with non-CMS cells under hypoxic conditions. This apparent discrepancy can be due to the erythroid progenitor differentiation stage and the severity of hypoxic culture conditions. The iPSC-derived CMS erythroid cells used were at the erythroid body stage II, which corresponds roughly to the proerythroblast to reticulocyte stage. The expression of EPOR increases from very low levels at the megakaryocyte-erythroid progenitor cell stage (1, 20), reaching a peak during the maturation from colony-forming units-erythroid (CFU-E) to proerythroblasts, and starts its decline during the progression into the remaining stages of the erythroid lineage (7, 23). Thus, less EPOR expression is expected at the proerythroblast and reticulocyte stage, compared with progenitors at earlier midstages of erythroid differentiation (BFU-Es and CFU-Es). The more severe hypoxic culture conditions used in the present study (1% vs. 5% \( O_2 \)) might also represent a stronger stimulus for EPOR expression. In line with the study on iPSCs, we did not find any expression difference in the \( EPO \) gene. Increased \( EPO \) expression has only been reported in mononuclear cells obtained from bone marrow biopsies of Han-Chinese CMS patients (48). Mononuclear cells require \( EPO \) for both commitment into the hematopoietic lineage and primary differentiation (19, 52, 55). Moreover, \( EPO \) is required to trigger EPOR expression in the later stages of erythroid differentiation (39, 42). Therefore, at this early stage, increased \( EPO \) expression and a paracrine \( EPO \) effect are required for differentiation, and if these events are more pronounced in CMS highlanders, they would contribute to the excessive RBC production. However, at BFU-E stage, less \( EPO \) is required to maintain intracellular \( EPO \) signaling and progenitor differentiation. Therefore, it makes sense that augmented erythroid proliferation of CMS BFU-E cells under hypoxic conditions relate to increased expression of upstream regulators such as \( SECP1 \), and to transcription factors such as \( GATA1 \) for upregulation of EPOR and other antiapoptotic factors. In the present study, we show that isolated native CMS

![Gene expression in CMS and non-CMS samples](image-url)
erythroid progenitors (CD34+ cells) produce a significantly larger relative proportion of late-stage erythroid precursors (CD235a cells) under hypoxic conditions, and that SENP1 knock-down brings differentiation and proliferation of erythroid precursors down to non-CMS levels. This observation confirms the key functional upstream role played by SENP1 in the augmented erythropoietic activity observed in native CMS cells.

Perspectives and Significance

Native CMS BFU-E erythroid progenitors show increased proliferative response under hypoxic conditions compared with non-CMS cells, and upregulated expression of SENP1, GATA1, and EPOR. Also, isolated native CMS CD34+ cells produce a more substantial proportion of erythroid precursors under hypoxia, and SENP1 knock-down abolishes this augmented proliferation, eliminating the cellular CMS phenotype. These findings suggest a significant intrinsic component at the cellular and gene expression level for the development of EE in CMS highlanders. Thus, our results are consistent with the possibility that this cellular EE phenotype might represent the basis for excessive RBC production, which could be further enhanced by poor systemic oxygenation during daytime or sleep as a consequence of altered respiratory control, or by differential hormonal patterns. The similarities between the hypoxic response of native CMS erythroid cells and the iPSCs-derived CMS erythroid lineage in terms of proliferation, differentiation, and gene expression validate the use of the latter to obtain further insight on the fundamental pathophysiological mechanism of EE. They also provide an unlimited resource to expand the search for novel cellular pathways and pharmacological targets with potential clinical applications on highlanders with this condition.

ACKNOWLEDGMENTS

We thank Renzo Gutierrez for assistance in part of the qRT-PCR experiments and Gianfranco Aicardi for technical assistance during fieldwork in Cerro de Pasco. We also thank the volunteers who took part in the study.

GRANTS

This work was funded by a Wellcome Trust Grant 107544/Z/15/Z to F. C. Villafuerte and by National Heart, Lung, and Blood Institute Grant 1R01HL146530-01 to GGH.
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DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

F.C.V. conceived and designed research; D.B., P.A., R.F.-M., G.V.-G., C.G.-G., and F.C.V. performed experiments; D.B., P.A., N.C., and F.C.V. analyzed data; D.B., P.A., N.C., and F.C.V. interpreted results of experiments; D.B., prepared figures; D.B., C.G.-G., and F.C.V. drafted manuscript; D.B., P.A., R.F.-M., G.V.-G., N.C., C.G.-G., G.G.H., and F.C.V. edited and revised manuscript; D.B., P.A., R.F.-M., G.V.-G., N.C., C.G.-G., G.G.H., and F.C.V. approved final version of manuscript.

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AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00250.2019 • www.ajpregu.org
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