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Basic nutritional investigation

Malnutrition suppresses cell cycle progression of hematopoietic progenitor cells in mice via cyclin D1 down-regulation

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ABSTRACT

Objective: Protein malnutrition (PM) often is associated with changes in bone marrow (BM) microenvironment leading to an impaired hematopoiesis; however, the mechanism involved is poorly understood. The aim of this study was to compare the cell cycle progression of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) and evaluate the cell cycle signaling in malnourished mice to assess the mechanism of cell cycle arrest.
Methods: C57Bl/6J mice were randomly assigned in control and malnourished groups receiving normoproteic and hypoproteic diets (12% and 2% protein, respectively) over a 5-wk period. Nutritional and hematologic parameters were assessed and BM immunophenotypic analysis was performed. Cell cycle of HPC (Lin^-) and HSC ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) were evaluated after 6 h of in vivo 5-bromo-2'-deoxyuridine (BrDU) incorporation. Cell cycle regulatory protein expression of HPC was assessed by Western blot.
Results: Malnourished mice showed lower levels of serum protein, albumin, glucose, insulin-like growth factor-1, insulin, and higher levels of serum corticosterone. PM also caused a reduction of BM myeloid compartment resulting in anemia and leukopenia. After 6 h of BrDU incorporation, malnourished mice showed G0-G1 arrest of HPC without changes of HSC proliferation kinetics. HPC of malnourished mice showed reduced expression of proteins that induce cell cycle (cyclin D1, cyclin E, pRb, PCNA, Cdc25a, Cdk2, and Cdk4) and increased expression of inhibitory proteins (p21 and p27) with no significant difference in p53 expression.
Conclusion: PM suppressed cell cycle progression mainly of HPC. This occurred via cyclin D1 down-regulation and p21/p27 overexpression attesting that BM microenvironment commitment observed in PM is affecting cell interactions compromising cell proliferation.

Introduction

As a definition, *malnutrition* may be taken as a subacute or chronic state of nutrition in which a combination of varying degrees of malnutrition and inflammatory activity lead to changes in body composition and physiological function [1]. Under these circumstances, the 2010 Food and Agriculture Organization report [2] estimated that globally 925 million people were malnourished, mostly in developing countries. However, several conditions apart from social establishments may lead to malnutrition. These include eating disorders, individuals with chronic disease, and long-term hospitalization [3–5], contributing to a worldwide public health problem. Clinically, the consequences of malnutrition have been associated with metabolic alterations, increased susceptibility to infections [6,7], and histologic and functional changes in several tissues, including bone marrow (BM) [8,9]. In fact, anemia and leukopenia reflect the hematopoietic tissue commitment in response to malnutrition and are associated with modification of the immune system that translates into increased susceptibility to infection [10–13], which is especially relevant to hospitalized individuals.

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Hematopoietic tissues show a high turnover rate, being extremely influenced by the availability of nutrients, because they are essential for cell cycle progression directly or indirectly [14]. Several studies highlight abnormalities in myelopoiesis and lymphopoiesis, followed by peripheral pancytopenia as a consequence of protein malnutrition (PM) [13,15–18]. We have demonstrated BM hypoplasia and structural breakdown with changes in the extracellular matrix (ECM) and stromal cells [8,9]. Cell cycle arrest of hematopoietic cells also has been observed with a delayed BM reconstitution after 5-fluoruracil treatment in malnourished mice [13,19].

There are two restricted populations of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) in BM that have the ability to generate all blood cell lineages in appropriate amounts to maintain system homeostasis. This is achieved due to strict cell cycle control mediated by extrinsic factors within BM and intrinsic factors at the cell level [20]. Therefore, cell interactions activate cell cycle signaling, promoting an orchestrated transition between G1-S-G2-M phases, which is regulated by cyclins and its effectors, cyclin-dependent kinase (Cdk) and cyclin-dependent kinase inhibitor (CKI) [21–24]. Thus, extracellular stimuli induces cyclin D1 expression and consequent cyclin D1/cdk4,6 complex formation responsible for the initial phosphorylation of retinoblastoma transcriptional repressors (pRb, p107, and p130), which keeps the *E2F* gene repressed. This initial phosphorylation promotes cyclin E synthesis and cyclin E/cdk2 complex formation, which further inactivates pRb-mediated inhibition of *E2F*, resulting in the G1-S transition. Meanwhile, members of the CKI family, namely inhibitors of kinase 4 (INK4: p15, p16, p19) and CDK interacting protein/kinase inhibitory protein (CIP/KIP: p21, p27) inhibit activation of the cyclin-cdk complexes, promoting a finer control of cell cycle progression [25–29].

Whereas the BM microenvironment commitment resulting from PM could compromise cell interactions, it is assumed that such a condition may blunt cyclin D1 expression, which in turn jeopardizes cell cycle initiation. To better understand the mechanisms involved in hematopoiesis impairment due to PM, we evaluated the effects of PM on HPC cell cycle signaling.

Materials and methods

Animals and diets

Two-mo-old male C57Bl/6J mice were obtained from the Animal Laboratory of Faculty of Pharmaceutical Sciences at the University of São Paulo. The mice (N = 26) were housed individually in metabolic cages (temperature controlled at 22 ± 2°C and a relative humidity of 55 ± 10% under a 12-h light/12-h dark cycle). After 2 wk of adaptation, the mice were randomly assigned to control (C) and malnourished (M) groups receiving normoproteic (12% protein) and a hypoproteic (2% protein) diets, respectively, over 5-wk periods. The murine diets (Table 1) were prepared in our laboratory according to the recommendations of the American Institute of Nutrition [30]. Mice were given free access to food and water. At the end of week 5, all mice were sacrificed under anesthesia with 10 mg/kg of xylazin (Rompum, Bayer, SP, Brazil) and 100 mg/kg of ketamine (Ketamina®, Cristália, SP, Brazil) between 0700 and 0900. The mice were fasted for 8 h before sacrifice. This study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Pharmaceutical Sciences (CEEA n° 92), University of São Paulo, and was conducted according to the guidelines of the Brazilian College on Animal Experimentation.

Table 1
Nutrient composition of the two experimental diets (g/1000 g of diet)

Ingredients	Normoproteic diet	Hypoproteic diet
Casein	120	20
Sucrose	100	100
Soybean oil	40	40
Fiber	50	50
Choline bitartrate	2.5	2.5
L-cystine	1.8	0.3
Tert-butylhydroquinone	0.008	0.008
Vitamin mixture	10	10
Mineral mixture	35	35
Cornstarch	640.692	742.192

Table 2
Nutritional and hormonal parameters

Parameters	Control (n = 8)	Malnourished (n = 10)	*P*-value
Body weight variation (%)	+27.2 ± 2.8	−5.6 ± 0.8‡	<0.0001
Food consumption (g/d/animal)	3.6 ± 0.1	4.5 ± 0.1‡	<0.0001
Protein consumption (g/d/animal)	0.44 ± 0.01	0.12 ± 0.04‡	<0.0001
Calorie intake (kcal/d)	13.95 ± 0.37	16.92 ± 0.48‡	<0.0001
Total serum protein (g/dL)	5.1 ± 0.4	4.2 ± 0.3*	0.0451
Serum albumin (g/dL)	2.0 ± 0.1	1.7 ± 0.1*	0.0268
Serum glucose (mg/dL)	101.8 ± 7.4	69.4 ± 4.0†	0.0014
Insulin concentration (ng/mL)	0.62 ± 0.09	0.24 ± 0.05†	0.0041
IGF-1 concentration (ng/mL)	546 ± 107	255 ± 420†	0.0066
Corticosterone concentration (ng/mL)	12.3 ± 0.1	40.2 ± 7.1*	0.0174

IGF, insulin-like growth factor
Data set analyzed by the Student's *t* test. Values are expressed as mean ± SD (*$P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$)

Nutritional and hormonal evaluation

The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The hormonal profile was evaluated by enzyme-linked immunosorbent assay (ELISA) quantification of serum insulin (Rat/Mouse Insulin ELISA, LINCO, St. Charles, MO, USA), insulin-like growth factor (IGF-1, Mouse IGF-1 Quantikine ELISA, R&D System, Minneapolis, MN, USA) and corticosterone (corticosterone EIA, IDS, Boldon, UK). The colorimetric analyses were assessed in a plate reader (EL800 Universal Microplate Reader - Instrumentals Bio-Tek Inc, Winooski, VT, USA).

Hematologic evaluation

Blood samples with EDTA (Sigma-Aldrich, St Louis, MO, USA) as anticoagulant were used for hemogram evaluation performed on ABX Micros ABC Vet® equipment (Horiba, Montpellier, France). Morphologic and leukocyte differentiation analyses were carried out on blood smears stained with standard May-Grunwald Giemsa technique. For reticulocyte identification, a supravital dye was used and quantification was performed on smear preparations. Total BM cells were harvested from both femur and tibia cavities using Iscove's medium (Sigma-Aldrich, St. Louis, MO, USA) and used for myelogram and immunophenotyping analyses. For myelogram, total cell counts were carried out using a Newbauer Chamber followed by differential counts using cytocentrifuge preparations stained with May-Grunwald Giemsa in which 300 cells per slide per animal were counted.

Table 3
Bone marrow cell counts

	Control (n = 6)	Malnourished (n = 6)	*P*-value
Total cells ($10^3/mm^3$)	40.7 ± 1.4	30.3 ± 1.5‡	<0.0001
Blast cells ($10^3/mm^3$)	1.0 ± 0.2	0.5 ± 0.1*	0.0445
Granulocyte precursors ($10^3/mm^3$)	2.7 ± 0.3	1.7 ± 0.1*	0.0304
Band/segmented cells ($10^3/mm^3$)	17.8 ± 0.6	13.6 ± 0.9*	0.0192
Eosinophil ($10^3/mm^3$)	0.5 ± 0.1	0.2 ± 0.1*	0.0149
Lymphocyte ($10^3/mm^3$)	8.4 ± 1.2	7.5 ± 0.7	0.5982
Macrophage ($10^3/mm^3$)	0.1 ± 0.0	0.1 ± 0.0	0.5200
Total erythroblast ($10^3/mm^3$)	9.3 ± 0.5	5.8 ± 0.3†	0.0052

Data set analyzed by the Student's *t* test. Values expressed as mean ± SD (*$P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$)