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Myelopoiesis modulation by ACE hyperfunction in kinin B_1 receptor knockout mice: Relationship with AcSDKP levels

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ABSTRACT

Angiotensin I-converting enzyme (ACE), a common element of renin–angiotensin system (RAS) and kallikrein–kinin system (KKS), is involved in myelopoiesis modulation, mainly by cleaving the tetrapeptide *N*-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP). Based on this finding and in our results showing B1 and B2 kinin receptors expression in murine bone marrow (BM) cells, we evaluated the ACE influence on myelopoiesis of kinin B1 receptor knockout mice (B1KO) using long-term bone marrow cultures (LTBMCs). Captopril and AcSDKP were used as controls. Enhanced ACE activity, expressed by non-hematopoietic cells (Ter-199$^-$ and CD45$^-$), was observed in B1KO LTBMCs when compared to wild-type (WT) cells. ACE hyperfunction in B1KO cells was maintained when LTBMCs from B1KO mice were treated with captopril (1.0 μM) or AcSDKP (1.0 nM). Although no alterations were observed in ACE mRNA and protein levels under these culture conditions, 3.0 nM of AcSDKP increased ACE mRNA levels in WT LTBMCs. No alteration in the number of GM-CFC was seen in B1KO mice compared to WT animals, even when the former were treated with AcSDKP (10 μg/kg) or captopril (100 mg/kg) for 4 consecutive days. Hematological data also revealed no differences between WT and B1KO mice under basal conditions. When the animals received 4 doses of lipopolysaccharide (LPS), a decreased number of blood cells was detected in B1KO mice in relation to WT. We also found a decreased percentage of Gr1$^+$/Mac-1$^+$, Ter119$^+$, B220$^+$, CD3$^+$, and Lin$^-$Sca1$^+$c-Kit$^+$ (LSK) cells in the BM of B1KO mice compared to WT animals. Low AcSDKP levels were observed in BM cultures from B1KO in comparison to WT cultures. We conclude that ACE hyperfunction in B1KO mice resulted in faster hydrolysis of AcSDKP peptide, which in turn decreased in BM tissues allowing HSC to enter the S stage of the cell cycle.

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1. Introduction

All blood cells can be generated from a common hematopoietic stem cell (HSC) through an extremely dynamic process called hematopoiesis or blood cell formation. Postnatal hematopoiesis takes place in the bone marrow (BM), where the HSCs as well as a complex mix of dividing and maturing cells of different lineages can be found [1,2]. Regulation of HSCs is governed by two intimately involved entities. One is the gene expression pattern in the cell and the other is the composition of external signals from the BM microenvironment. Transcription factors are internal signals that regulate gene expression, while external signals from the BM microenvironment can be mediated by cell–cell interactions, cell–extracellular matrix (ECM) interactions, and soluble growth factors secreted from hematopoietic and stromal cells [3–5].

Apart from transcription factors, there are also other intrinsic factors within the HSCs that are important regulators of stem cell fate. These include effector molecules in the cell cycle machinery. For instance, high levels of cyclin D_2 have been detected in cycling HSCs, suggesting an important role in HSC cycling, whereas studies of knockout mice have shown that the cyclin-dependent kinase inhibitor (CDKI) p21 acts to maintain HSCs in a quiescent state [6,7]. Signals from internal and external regulatory factors decide whether the HSCs are maintained in quiescence,

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proliferate, undergo apoptosis, or migrate out of the BM space [8].

Haznedaroğlu et al. [9] proposed that other mechanisms are also involved in hematopoiesis regulation. They hypothesized that a locally active, intrinsic renin–angiotensin system (RAS) exists in the BM, where it affects production, proliferation, and differentiation of the cells in both normal and malignant hematological processes. In accordance with this preliminary evidence, Strawn et al. [10] verified that all RAS components are detectable in normal BM derived blood cells at molecular and protein levels. In line with these studies, angiotensin-II type 1a (AT1a) receptors are present in $CD34^+$ hematopoietic cells and stimulate the proliferation of both BM and umbilical cord blood hematopoietic progenitors in physiological or pathological states [11–16]. Part of these events is modulated by the angiotensin I-converting enzyme (ACE), which also cleaves the negative physiological hematopoietic regulator *N* acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) into its inactive form [10,12,17,18]. Therefore, ACE hyperfunction may lead to the acceleration of AcSDKP metabolism, which in turn lowers its levels in the BM microenvironment, finally removing the antiproliferative effects of the peptide on the hematopoietic cells and blasts. The hemoregulatory tetrapeptide AcSDKP, originally isolated from fetal calf BM [19,20], reversibly prevents the recruitment of pluripotent HSCs and normal early progenitors into the S stage of the cell cycle by maintaining them quiescent.

Zambidis et al. [21], in an elegant study, demonstrated the role of renin–angiotensin axis in directly regulating human embryonic angio-hematopoietic genesis and also proved that captopril inhibition of ACE activity severely inhibited human hemangioblast colony expansion, similarly to the effects on progenitors described for murine BM cultures [11].

ACE and angiotensin-II (Ang II) receptor inhibition is also associated with improved HSC recovery after chemotherapy induction, an effect that was originally interpreted to be due to the tetrapeptide AcSKDP [22]. Since the Ang II peptide is a direct mitogen for both human erythroid and $CD34^+$ hematopoietic cell progenitors [23,12], and specific AGTR1 inhibition has been reported to favor hematopoietic progenitor expansion through an unknown mechanism [24], the modulator activities of ACE, AcSDKP, and Ang II in hematopoiesis may also be complicated by the involvement of bradykinin, another important peptide cleaved by ACE into its inactive form.

The kallikrein–kinin system (KKS) acts via two types of receptors: B1 and B2. B2 receptors are widely distributed in the vasculature and expressed constitutively, whereas B1 receptors are weakly expressed under physiological conditions, but strongly induced by pathological stimuli such as inflammation or tissue injury [25,26]. In general, activation of the B1 receptor produces prolonged, sustained, or oscillatory responses. This persistent signaling appears due to lack of desensibilization and internalization of B1 receptor [27–29]. Bradykinin (BK) is a potent inflammatory agent, which is an enzymatically produced peptide generated at inflammatory sites by the action of kallikreins [30,31]. This peptide exerts a variety of effects measurable as smooth muscle contraction, increased vascular permeability, vasodilation, and pain [32]. BK sensitization of myeloid progenitors to prostaglandin (PGE) further suggests that this peptide may act to influence myelopoiesis [32].

Intrigued by the possible role of RAS and KKS in myelopoiesis and based on the fact that ACE is a key component of both systems, using kinin B1 receptor knockout mice (B1KO) we evaluated ACE activity and the possible role of AcSDKP in murine BM cell proliferation. Cellular localization and BM cell populations involved in ACE expression were also assessed. For this purpose, throughout the experiments we used captopril and AcSDKP respectively as positive controls of ACE inhibition and ACE substrate.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated, and cell culture media and supplements were purchased from Gibco-BRL (Bethesda, MD, USA). ACE fluorescence resonance energy transfer substrate Abz-FRK(Dnp)P-OH was generously supplied by the Biophysics Department of the Universidade Federal de São Paulo (UNIFESP, São Paulo, Brazil).

2.2. Animals

This study was approved by an Institutional Ethics Committee from Mogi das Cruzes University (CEMEA/UMC), Mogi das Cruzes, SP, Brazil. All experiments reported were carried out as stated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals Committee. The experiments were carried out with kinin B1 receptor knockout mice (B1KO) and C57Bl/6 wild-type (WT) animals were used as controls. All the animals were obtained from the Centro de Desenvolvimento de Modelos Experimentais para a Medicina e Biologia at the UNIFESP and housed in plastic cages, in groups of five animals, in air-conditioned rooms at $22 \pm 2\,^{\circ}C$, with a 12-h light–dark natural cycle and allowed *ad libitum* access to food and tap water. The animals were euthanized by cervical dislocation, their femurs and spleens were dissected out, and the femur cellularity was approximately 1×10^7 nucleated cells. For *in vivo* studies, WT and B1KO mice ($n = 5$) were daily treated intraperitonially (ip) with captopril (100 mg/kg) or AcSDKP (10 μg/kg) [33] for 4 consecutive days and 24 h after the last dose, BM cells were removed by flushing to be used in the granulocyte-macrophage colony-forming cell (GM-CFC) assay, which was conducted as described in Section 2.4. Spleen weight of B1KO and WT mice was evaluated as well.

To carry out hematological studies, WT and B1KO mice ($n = 7$) were injected (i.p.) with lipopolysaccharide (LPS *E. coli*, 0111 : B4) for 4 consecutive days (3 days with the dose of 400 μg/kg and one last dose of 14 mg/kg). or saline and leucocyte, erythrocyte, hemoglobin, hematocrit, and platelet levels were counted by standard methods 24 h after the last dose of LPS administration. Giemsa stained blood smears and a total of 100 cells were counted.

2.3. Establishment of long-term bone marrow cultures (LTBMCs) from WT and B1KO mice

Femurs excised from WT and B1KO mice were flushed with Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% horse serum (HS), 50 international units (IU) benzyl penicillin, 50 μg/mL streptomycin, 2.0 mM L-glutamine, and 10^{-6} M hydrocortisone into 6-well tissue culture plates. Half of the medium in each 6-well plate was replaced weekly with an equal amount of fresh medium. At the end of the fourth week, after stroma formation, new BMs from other mice were collected in supplemented IMDM and cultured (2 h) in tissue culture flasks (75 cm^2). Non-adherent cells were collected by removing the medium and 10^6 cells/well were added to pre-cultured stroma. The cultures were submitted to *in vitro* treatment receiving captopril (1.0 μM), or AcSDKP (1.0 nM), or DBK (1.0 μM), or Lys-bradykinin (LBK) (1.0 μM) at the beginning of the culture.

2.4. Granulocyte-macrophage colony-forming cell (GM-CFC) assay

Cells from BM of mice treated with captopril or AcSDKP (10^5 cells/mL) for 4 consecutive days were suspended in Dulbecco's medium supplemented with 20% HS, 50 IU benzyl penicillin,

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