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PTEN is indispensable for cells to respond to MAPK inhibitors in myeloid leukemia

Jingliao Zhang[a,b,1], Zhifu Xiang[a,1], Priyangi A. Malaviarachchi[a], Yan Yan[a], Nicholas J. Baltz[a], Peter D. Emanuel[a,*], Y. Lucy Liu[a,*]

[a] Winthrop P. Rockefeller Cancer Institute, Division of Hematology, Department of Internal Medicine, College of Medicine, University of Arkansas for Medical Sciences (UAMS), Little Rock, AR 72205, United States
[b] Department of Pediatrics, Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences, Tianjin 300020, China

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ABSTRACT

Constitutively activated MAPK and AKT signaling pathways are often found in solid tumors and leukemias. PTEN is one of the tumor suppressors that are frequently found deficient in patients with late-stage cancers or leukemias. In this study we demonstrate that a MAPK inhibitor, PD98059, inhibits both AKT and ERK phosphorylation in a human myeloid leukemia cell line (TF-1), but not in PTEN-deficient leukemia cells (TF-1a). Ectopic expression of wild-type *PTEN* in myeloid leukemia cells restored cytokine responsiveness at physiological concentrations of GM-CSF (< 0.02 ng/mL) and significantly improved cell sensitivity to MAPK inhibitor. We also found that Early Growth Response 1 (EGR1) was constitutively over-expressed in cytokine-independent TF-1a cells, and ectopic expression of *PTEN* down-regulated EGR1 expression and restored dynamics of EGR1 expression in response to GM-CSF stimulation. Data from primary bone marrow cells from mice with *Pten* deletion further supports that PTEN is indispensible for myeloid leukemia cells in response to MAPK inhibitors. Finally, We demonstrate that the absence of EGR1 expression dynamics in response to GM-CSF stimulation is one of the mechanisms underlying drug resistance to MAPK inhibitors in leukemia cells with PTEN deficiency. Our data suggest a novel mechanism of PTEN in regulating expression of EGR1 in hematopoietic cells in response to cytokine stimulation. In conclusion, this study demonstrates that PTEN is dispensable for myeloid leukemia cells in response to MAPK inhibitors, and PTEN regulates EGR1 expression and contributes to the cytokine sensitivity in leukemia cells.

1. Introduction

The mitogen-activated protein kinase (MAPK) signaling pathway is activated by many extra- and intracellular stimuli, including cytokines and growth factors [1]. This pathway plays a key role in regulation of cellular processes, including proliferation, differentiation, response to stresses, motility, survival, and death [2]. Constitutively activated MAPK and AKT pathways are often found in leukemia [3]. We previously reported that patients with juvenile myelomonocytic leukemia (JMML), which is an aggressive pediatric leukemia with myeloid cells selectively hypersensitive to granulocyte-macrophage colony-stimulating factor (GM-CSF), had constitutive-hyperactive AKT and MAPK [4]. Therefore, inhibition of MAPK pathway might serve as an attractive therapeutic strategy for leukemia treatment. However, current progress in the development of MAPK inhibitors as therapeutics for leukemia has been disappointing. One of the reasons for the slow progress may be related to the fact that these agents were typically first evaluated in patients with advanced diseases. Unfortunately, many of these patients' tumor cells had gained additional genetic or epigenetic abnormalities, over-activated other signaling pathways, and developed tumor suppressor gene deficiencies. Importantly, patients who participated in those prior clinical trials might not be comprehensively evaluated for their genetic and/or molecular profiles. As a consequence, this might lead to inaccurate conclusions regarding the efficacy of some new investigational drugs. Therefore, besides fundamentally understanding the biology of late-stage cancers and leukemia, it is critical to incorporate information on genetic/epigenetic and cell signaling transduction into the development of new therapeutics to ensure accurate conclusion from evaluation of new investigational drugs.

PTEN, as a tumor suppressor, negatively regulates both PI3K-AKT-

* Corresponding authors at: Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, 4301 W. Markham St., Slot #753, Little Rock, AR 72205, United States.
E-mail addresses: PDEmanuel@uams.edu (P.D. Emanuel), ylucyliu@uams.edu (Y.L. Liu).
[1] These authors contributed equally to this work.

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dependent and -independent signaling pathways, such as the MAPK pathway, which control multiple cellular functions, including cell growth, survival, proliferation, and migration in a context-dependent manner [5, 6]. Previous studies have shown that intact PTEN function is essential for normal hematopoiesis in maintaining hematopoietic stem cell (HSC) pool, activating HSCs, governing lineage determination, and preventing leukemia transformation [7–9]. PTEN loss is frequently found in patients with late-stage cancers or leukemia. Loss of PTEN function leads to increased PI3K and MAPK signaling, resulting in hyperplasia and tumorigenesis. In mice, somatic deletion of *Pten* gene in myeloid tissues results in enhanced cell proliferation and depletion of HSC population in bone marrow, and also causes myeloid leukemia and myeloproliferative neoplasms [9–11]. We reported that leukemic mice with *Pten* deletion (*PtenΔ/Δ*) in myeloid tissue had predominantly hyperactive MAPK activities [11]. The genetic abnormality of *PTEN* in human *de novo* myeloid malignancies is uncommon. However, functional loss of PTEN through transcriptional regulation, post-transcriptional regulation by non-coding RNAs, phosphorylation-mediated inactivation, delocalization, and other mechanisms have been reported [5, 12–14]. We previously reported PTEN protein deficiency in 67% of JMML patients, which was partially related to DNA hypermethylation on *PTEN* promoter [4]. Although cytokine-independency is often found in leukemia cells, it is unclear how PTEN regulates the cell responsiveness to cytokine in hematopoietic cells. We hypothesized that PTEN deficiency might cause insufficient negative signaling controls to counter the hyperactive MAPK and AKT signaling pathways that are caused by mutations, resulting in cytokine-hypersensitivity or independency in leukemic cells. Recent data suggested that PTEN status was crucial for the inhibition of MEK and mTOR inhibitors in solid cancer cell lines *in vitro* [15], and loss of PTEN expression has been reported related to drug resistance in lung cancer cells [16]. But it is unknown whether PTEN loss has any impact on leukemia cells in response to MAPK inhibitors. In the present study, we demonstrate that PTEN contributes to cellular responsiveness to GM-CSF and Interleukin 3 (IL-3), and PTEN expression is required for cells to effectively respond to MAPK inhibitors. Furthermore, we demonstrate that a sufficient PTEN expression is required for the intact dynamics of EGR1 expression in response to GM-CSF stimulation in myeloid cells.

2. Material and methods

2.1. Cell culture

The growth factor-dependent human myeloid cell line TF-1 (ATCC®) and the growth factor-independent cell line TF-1a (ATCC®) were maintained in RPMI-1640 medium (ATCC®) supplemented with 10% fetal bovine serum (FBS) in the presence or absence of human recombinant GM-CSF (rhGM-CSF) and IL-3 (rhIL-3) (R & D Systems) at concentration of 2 ng/mL at 37 °C in humidified air containing 5% CO_2. For evaluation of cytokine responsiveness, cells were serum-starved in RPMI-1640 with 0.5% BSA for 16 h, followed by stimulation with rhGM-CSF or rhIL-3 at desired concentrations and desired time. In experiments testing for MAPK and AKT inhibition, cells were treated with culture medium containing 100 μM of PD98059 (Calbiochem), or 10 μM of LY29004 (Calbiochem), or 0.01% DMSO (Sigma) for 1 h before cytokine stimulation.

2.2. Retroviral wild-type PTEN constructs and cell transformation

Wild-type human *PTEN* coding sequence was cut out from *pLNCX-PTEN* (kindly provided by Dr. Muxiang Zhou from Emory University) and was sub-cloned into *pBMN-GFP* retroviral expression vector (Orbigen, Inc.). Retroviral supernatant was produced in 293 T cell line (Phoenix™) following the manufacturer's instructions. TF-1a cells were transfected with retrovirus containing *pBMN-PTEN-GFP* or *pBMN-GFP* by spinoculation following the manufacturer's instructions. Transfected cells expressing GFP were sorted by fluorescence-activated cell sorting (FACS) on an Aria II cell sorter (BD Biosciences). Single cells were seeded and expanded in each well of a 96-well plate containing RPMI-1640 (ATCC®) supplemented with 10% FBS and rhGM-CSF and rhIL-3 at concentrations of 2 ng/mL.

2.3. Colony formation assay

Colony formation assays were performed as previously reported with modifications [4]. Briefly, triplicate 1-mL semi-solid cultures containing 2000 cells/mL were established in 35-mm dishes containing 0.3% agar and McCoys' 5A medium (Sigma) supplemented with 15% FBS and nutrients in the presence or absence of rhGM-CSF or rhIL-3 at desired concentrations. For inhibition experiments, one hundred microliters of culture medium containing vehicle control (0.01% DMSO) or MAPK inhibitor PD0325901 (Selleckchem) at various concentrations were added to each dish next day after plating. After 14 days of incubation in 5% CO_2 at 37°C, colonies with aggregates of > 40 cells per cluster were counted and scored. The cytokine sensitivities of cells were presented as the percentiles of the colony number at each concentration of a cytokine relative to the maximal colony numbers at a saturated concentration (0.16 ng/mL), in order to limit the variations from day-to-day and person-to-person.

2.4. Proliferation assay

Bone marrow (BM) nucleated cells from mice with somatic *Pten* deletion (*PtenΔ/Δ*) in myeloid tissues or wild type were prepared as previously reported [11]. Briefly, 5×10^4 cells in 50 μL of culture medium RPMI-1640 supplemented with 10% FCS, 2 ng/mL of recombinant mouse GM-CSF (rmGM-CSF) and IL-3 (rmIL-3) (R & D Systems) were seeded in triplicate onto 96-well plates. Fifty microliters of culture medium containing vehicle control (0.01% DMSO) or MAPK inhibitor PD0325901 in desired concentrations were added to each respective well after cells were settled for 30 min. After an average 72-h culture in 5% CO_2 at 37°C, 10 μL of alamarBlue™ (Life Technologies) was added to each well. The plates were read on a plate reader (ELx800, BioTek) after 4-h incubation in incubator. The proliferation rate was calculated according to the protocol recommended by manufacturer. The experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

2.5. Semi-quantitative RT-PCR

Total RNA extraction, cDNA synthesis, and semi-quantitative RT-PCR were performed as previously reported [17]. Briefly, total RNAs were extracted using Trizol solution following the manufacturer's instructions (Life Technologies). Two hundreds nanograms of total RNAs were used as templates for first strand cDNA synthesis using SuperScript II kit (Life Technologies). Semi-quantitative RT-PCR was performed using Invitrogen™Ambion™QuantumRNA™ with 18S Internal Standards kit (Life Technologies). PCR products were visualized by electrophoresis on a 1.5% agarose gel stained with SyBr Gold™ (Life Technologies). The individual band intensity of PCR bands were documented by a Molecular Imager® ChemiDoc™XRS (Bio-Rad). The sequences of the primers used in semi-quantitative RT-PCR for *EGR1* were: 5′-ATC CCC GAC TAC CTG TTT CC-3′ (forward); and 5′-CCG CAA GTG GAT CTT GGT AT-3′ (reverse).

2.6. Western blot

Mouse BM nucleated cells or cell line cells were serum-starved in RPMI-1640 with 0.5% BSA for 4 h or 16 h, respectively, followed by stimulation with desired concentrations of GM-CSF or IL-3. Cells were collected at desired time points after stimulation and washed twice with

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