LETTER TO THE EDITOR

p27KIP1 and PTEN cooperate in myeloproliferative neoplasm tumor suppression in mice

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Abstract

PTEN acts as a phosphatase for PIP3 and negatively regulates the PI3K/AKT pathway, and p27KIP1 is a cyclin-dependent kinase inhibitor that regulates the G1 to S-phase transition by binding to and regulating the activity of cyclin-dependent kinases. Genetic alterations of PTEN or CDKN1B (p27KIP1) are common in hematological malignancies. To better understand how mutations in these two genes might cooperate in leukemogenesis, we inactivated both genes in the hematological compartment in mice. Here, we show that the combined inactivation of Pten and Cdkn1b results in a more severe myeloproliferative neoplasm phenotype associated with lower hemoglobin, enlarged spleen and liver, and shorter lifespan compared to inactivation of Pten alone. More severe anemia and increased myeloid infiltration and destruction of the spleen contributed to the earlier death of these mice, and elevated p-AKT, cyclin D1, and cyclin D3 might contribute to the development of this phenotype. In conclusion, PTEN and p27KIP1 cooperate in tumor suppression in the hematological compartment.

Keywords: PTEN, p27KIP1, Myeloproliferative neoplasms

Background

PTEN (phosphatase and tension homolog deleted on chromosome 10) is a tumor suppressor gene located on chromosome 10q23 and is one of the most commonly mutated or deleted genes in human cancers, including acute lymphoblastic leukemia, juvenile myelomonocytic leukemia, and non-Hodgkin’s lymphoma [1, 2]. PTEN acts as a phosphatase for phosphatidylinositol-3,4,5-trisphosphate (PIP3) and negatively regulates the phosphatidylinositol 3-kinase (PI3K)/AKT pathway [3]. The CDKN1B gene encodes p27KIP1, which belongs to the Cip/Kip family of cyclin-dependent kinase inhibitors. p27KIP1 is a key regulator of the G1 to S-phase transition by inhibiting cyclinD1/CDK4 and cyclinE/CDK2 complexes [4]. Deletions and other cytogenetic aberrations involving CDKN1B have been reported in a variety of leukemias [5–7]. In addition, CDKN1B expression can be a useful prognostic molecular marker for acute myeloid leukemia, where low CDKN1B expression is associated with high proliferation and, therefore, with a favorable response to chemotherapy [6]. Inactivation of the tumor-suppressor gene PTEN and lack of CDKN1B expression have been detected in some kinds of cancer, including most advanced prostate cancers and lymphomas [8, 9]. It has been shown that the combined loss of PTEN and p27KIP1 is associated with tumor cell proliferation and increased risk of recurrent disease in localized prostate cancer [10]. Loss of PTEN expression is more frequent in anaplastic large-cell lymphoma, which strongly correlates with the loss of CDKN1B expression [9].

Targeted disruption of the murine Cdkn1b gene causes a gene dose-dependent increase in animal size without other gross morphologic abnormalities [11], and deletion of Pten in the hematopoietic compartment in mice promotes excessive proliferation of leukemogenic stem cells resulting in the development of myeloproliferative neoplasms [12].
neoplasm (MPN) followed by acute leukemia [12]. In mice, concomitant inactivation of Pten and Cdkn1b accelerates spontaneous neoplastic transformation of prostate cancer [8]. In order to better understand the relation and clinical relevance of these two genes in the pathogenesis of hematological malignancies, we used Cre recombinase to simultaneously inactivate Pten and Cdkn1b in the hematopoietic compartment.

Results and discussion

To determine the impact of combined deficiency of PTEN and p27KIP1 in the hematopoietic compartment, we injected pI–pC into PCM, PM, CM, and Ctrl mice. Consistent with previous studies [13], all PM mice died from MPN by 98 days after pI–pC injections (median survival 62 days), whereas CM and Ctrl mice lived much longer and no MPN phenotype was observed in CM mice. However, the maximum survival of PCM mice was only 30 days (median 24 days; \( p < 0.001 \) versus PM; Fig. 1a).

Two weeks after pI–pC injections, white blood cell counts were 20.8 × 10⁹ cells/L in PCM mice compared with mean counts of 18.3 × 10⁹, 13.9 × 10⁹ and 13.6 × 10⁹ cells/L for PM, CM and Ctrl mice, respectively (Fig. 1b). However, no morphological changes and no increase in the amounts of immature cells, including myeloblasts, could be detected in the bone and bone marrow in PCM mice compared with the other three groups (Fig. 1c, e). More severe anemia and more architectural disruption of the spleen were observed in PCM mice (Fig. 1d, e).

Spleen and liver weights in PCM mice increased by 2.3–5.6 and 1.2–2.4-fold, respectively, compared with the other three groups (Fig. 2d). No increased colony formation was observed in the bone marrow of PCM mice compared with the other three groups (Fig. 2g). In conclusion, our results show that PTEN deficiency can promote tumor progression by a decrease in p27KIP1 levels in the hematological compartment and that PTEN and p27KIP1 have a cooperative role in leukemia suppression. In addition, our results show that elevated phosphorylated AKT, cyclin D1, and cyclin D3 might play an important role in the progression of the severe MPN phenotype.

Methods

Animal procedures

Mice with conditional Pten⁰⁄⁰ alleles (designated P) with a mixed genomic background of 129S4/SvJae and C57BL/6J were bred with Cdkn1b⁰⁄⁰ mice (designated C) to generate PC mice. PM mice were bred with mice harboring the interferon (IFN)-inducible Mx1-Cre transgene (designated M) to generate PCM mice (Pten⁰⁄⁰ Cdkn1b⁰⁄⁰ Mx1-Cre), PM (Pten⁰⁄⁰ Mx1-Cre), and CM (Cdkn1b⁰⁄⁰ Mx1-Cre) mice. Mice without Mx1-Cre were used as healthy controls (designated Ctrl).

The mice were housed under controlled environmental conditions with free access to water and food. Illumination was on between 0600 and 1800 h. All mice were monitored daily. Groups of 4-week-old mice were injected with 400 µg polyinosinic-polycytidylic acid (pI–pC; Sigma, St Louis, MO). Blood was taken weekly and analyzed with a hematology analyzer KX-21 (Sysmex Europe, Norderstedt, Hamburg, Germany). Three weeks after injection, groups of mice were sacrificed and their tissues were harvested for further analysis. Mice were euthanized by cervical dislocation after carbon dioxide inhalation. In addition, groups of mice were kept for a survival study. If mice had ruffled fur and became listless...
or lost more than 10 % of their body weight, they were euthanized. All experimental protocols were approved by the regional ethical committee of the University of Gothenburg, Sweden.

**Genotyping**

Genotyping was performed by PCR amplification of genomic DNA extracted from mouse tails. The Pten<sup>fl</sup> allele was detected with forward primer 5'-CAAG CACTCTGCAACTGAG-3' and reverse primer 5'-AA GTTTTTGAAAGCAAGATGC-3', yielding a 328-bp fragment from the Pten<sup>fl</sup> allele and a 156-bp fragment from the Pten<sup>+</sup> allele. The P27<sup>fl</sup> allele was detected with forward primer 5'-TAGGGGAAAT GGATAGTAGATGTTAGGACC-3' and reverse primer 5'-GTTTAAATATGGAAGTGAATCTCAATGG CC-3', yielding a 400-bp fragment from the P27<sup>fl</sup> allele and a 370-bp fragment from the P27<sup>+</sup> allele. The Mx1-Cre transgene was detected with forward primer 5'-GCGGTCTGGCAGTAAAAACTATC-3' (oIMR 1084) and reverse primer 5'-GTGAAACAGCATTGCTGTC ACTT-3' (oIMR 1085) to yield a 100 bp fragment.

**Fluorescence-activated cell sorting, colony assays, and histology**

Splenocytes and bone marrow cells were incubated with antibodies against Gr1 (PE-Cy7/RB6-8C5), CD11b (V450/M1/70), c-kit (PE/Cy7/D7), Lin- (FITC) and CD45 (V500/30-F11) and analyzed with FACS Diva software (BD Biosciences, San Jose, CA, USA). For colony assays, splenocytes (1 × 10<sup>5</sup>) and bone marrow cells (2 × 10<sup>5</sup>) harvested from experimental mice were seeded in duplicate wells in methylcellulose medium (MethoCult M3434; StemCell Technologies, Vancouver, BC, Canada). Six days later, the numbers of colonies were scored. For bone marrow cells, on the 7th day the cultured cells were washed, collected, and replated. Histology was performed as described [19, 20].
Western blots

Tissue pieces (50–100 mg) were lysed in ice-cold buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% NP-40, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 mM orthovanadate, and the Complete Mini protease inhibitor cocktail). Lysates were homogenized, and centrifuged at 20,000g for 20 min, and equal amounts of total protein of the supernatant were size-fractionated on 10–15% sodium dodecyl sulfate polyacrylamide gels. The proteins were transferred onto nitrocellulose membranes and incubated with antibodies against phosphorylated ERK1/2 (9106), total ERK (9102), phosphorylated AKT (9271), PTEN (9559), p27KIP1 (2552; Cell Signaling,
Danvers, MA), Cyclin D1(sc-718), Cyclin D3 (sc-182), and Beta-actin (sc-7778; Santa Cruz Biotechnology, Inc., Dallas, TX). Protein bands were visualized with a horse-radish peroxidase-conjugated secondary antibody (170-5046 and 170-5047; Bio-Rad Laboratories, Inc., Hercules, California) and the Enhanced Chemiluminescence Kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Band density was measured by Quantity One software (Bio-Rad Laboratories, Inc. USA).

Statistical analyses
Data are plotted as the mean ± SEM. Differences in the concentrations and percentages of white blood cells, the colony-forming ability of hematopoietic cells, and the proliferation of cells in culture were determined with Student’s t test. Differences in mouse survival were assessed by the Mann–Whitney U test.

Abbreviations
PTEN: phosphatase and tensin homolog; CDKN1B: cyclin-dependent kinase inhibitor 1B; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT(PKB): protein kinase B; PIP3: phosphatidylinositol-3,4,5-trisphosphate; MPN: myeloproliferative neoplasm; pI–pC: polyinosinic–polycytidylic; PCM: Ptenfl/fl; LSK: lineage-negative (lin−), Sca-1+ Mx1-Cre; PM: Ptenfl/fl Mx1-Cre; CM: Cdkn1Bfl/fl Mx1-Cre; Ctrl: Control; colony-forming ability of hematopoietic cells, and the

Authors’ contributions
JS designed and carried out experimental work and wrote the manuscript. VML designed the work and wrote the manuscript. SL carried out experimental work and analysis of data. KB carried out the acquisition and analysis of data. SYW, KL analysed the data and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
Not applicable.

Consent for publication
Not applicable.

Ethics approval and consent to participate
All animal experimental protocols were approved by the regional ethical committee of the University of Gothenburg, Sweden.

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