Effects of purine nucleoside phosphorylase deficiency on thymocyte development

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Background: Inherited or acquired defects in purine nucleoside phosphorylase (PNP) impair purine metabolism, as well as the survival and function of T lymphocytes. However, the effects of PNP deficiency on thymocyte development are not well known.
Objectives: We sought to study thymocyte development in PNP-deficient (PNP-KO) mice.
Methods: Maturation, proliferation, and apoptosis were determined in thymocytes from PNP-KO mice and hematopoietic stem cells from these mice grown *ex vivo* into thymocyte-like cells.
Results: Reduced percentages of $CD4^+CD8^+$ double-positive (DP) thymocytes with normal percentages of $CD4^-CD8^+$ and $CD4^+CD8^-$ single-positive thymocytes were found in the thymi of PNP-KO mice. Similarly, reduced DP-like thymocytes grew *ex vivo* from hematopoietic stem cells of PNP-KO mice. Thymi of PNP-KO mice contained increased apoptotic DP thymocytes. Increased apoptosis of PNP-deficient DP thymocytes occurred after exposure to deoxyguanosine (dGuo), although not after Fas ligation, and could be prevented by restoring PNP activity within the cells. In DP thymocytes from PNP-KO mice, dGuo caused mitochondrial membrane potential dissipation and induced release of cytochrome c from the mitochondria followed by nuclear DNA fragmentation. Inhibition of the caspase pathway prevented dGuo-induced nuclear DNA fragmentation but not mitochondrial membrane potential dissipation, indicating that PNP deficiency induces apoptosis that is initiated in the mitochondria of DP thymocytes. 5-Bromo-2-deoxyuridine incorporation demonstrated that PNP deficiency does not interfere with DP or single-positive thymocyte proliferation.
Conclusions: PNP is important for the survival of DP thymocytes. Accumulation of dGuo in cases of PNP deficiency leads to mitochondria-initiated apoptosis of DP thymocytes, which can be prevented by restoring PNP activity in the cells. (J Allergy Clin Immunol 2011;128:854-63.)

Key words: *Purine nucleoside phosphorylase, thymocytes, apoptosis, proliferation, mice, immune deficiency*

Purine nucleoside phosphorylase (PNP) is an intracellular enzyme responsible for the phosphorolysis of deoxyguanosine (dGuo), which can be further metabolized to uric acid or salvaged into the nucleotide pools (Fig 1, *A*). Inherited defects in PNP function cause accumulation of dGuo in the cells, which can be phosphorylated to deoxyguanosine monophosphate (dGMP), deoxyguanosine diphosphate, and deoxyguanosine triphosphate (dGTP) by the mitochondrial deoxyguanosine kinase (dGK) enzyme.[1] Excess dGTP is particularly toxic to lymphocytes, and therefore synthetic PNP inhibitors, such as forodesine, have been developed that affect lymphocyte survival and function.[2] Studying peripheral blood lymphocytes from patients with chronic lymphocytic leukemia treated with forodesine suggested that PNP has a role in preventing mitochondria-induced apoptosis.[2] Also, analysis of thymocytes lacking adenosine deaminase (ADA), another purine enzyme important for lymphocyte development and function, has indicated that abnormal purine homeostasis can cause increased mitochondria-induced apoptosis.[3] Understanding the functions of PNP in thymocytes is particularly important for the management of PNP-deficient patients, as well as for the many patients with malignancy and immune dysregulation conditions who are treated with synthetic PNP inhibitors.[4] However, the effects of PNP deficiency on thymocytes have not been thoroughly investigated.

Analysis of PNP-deficient thymocytes have been hampered by the limited availability of thymi from PNP-deficient patients and the confounding effects that prolonged infections, malnutrition, or immunomodulating agents might have on thymi obtained from autopsies. To better appreciate the functions of PNP, we previously knocked out the catalytic domain of PNP in mice and showed that PNP-deficient (PNP-KO) mice recapitulated many of the metabolic and immune abnormalities seen in PNP-deficient patients.[5,6] The urine and sera of PNP-KO mice contained increased dGuo concentrations, whereas thymocytes had increased dGTP concentrations, particularly in the mitochondria. PNP-KO mice exhibited a profound decrease in the number and function of peripheral T lymphocytes, as well as marked perturbations in thymocytes. In addition, we showed that dGuo could induce apoptosis in thymocytes from normal (PNP-proficient) mice treated *ex vivo* with synthetic PNP inhibitor and that the apoptosis was initiated in the mitochondria.[5] However, these studies were not performed in PNP-deficient thymocytes, and induction of apoptosis in these cells required

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Abbreviations used

ADA: Adenosine deaminase
BrDU: 5-Bromo-2-deoxyuridine
dGK: Deoxyguanosine kinase
dGMP: Deoxyguanosine monophosphate
dGTP: Deoxyguanosine triphosphate
dGuo: Deoxyguanosine
DN: Double negative
DP: Double positive
FACS: Fluorescence-activated cell sorting
HSC: Hematopoietic stem cell
MMP: Mitochondrial membrane potential
OP9-DL1: OP9 bone marrow stromal cells expressing the delta-like ligand 1
PI: Propidium iodide
PNP: Purine nucleoside phosphorylase
PNP-KO: PNP deficient
SP: Single positive
TAT-PNP: Purine nucleoside phosphorylase fused to TAT protein transduction domain
TCR: T-cell receptor

excessive dGuo concentrations; therefore the relevance of our findings to thymocyte development in PNP deficiency was questioned.

Thymocyte development in mice is a well-defined process. $CD4^-CD8^-$ double-negative (DN) cells reside in the thymus cortex and constitute 1% to 3% of the thymocytes in normal adult mice. As DN thymocytes transit through the cortex toward the medulla, they rearrange their V(D)J genes and eventually express a diverse T-cell receptor (TCR) repertoire. Many molecules influence maturation of DN cells, including Bim, which has been found to have an important role in physiological thymopoiesis. Indeed, in the absence of Bim, thymocyte maturation and proliferation are severely disrupted.[7] TCR expression by thymocytes is followed by thymocyte proliferation and upregulation of CD4 and CD8 expression on thymocytes.[8] Cells that express both CD4 and CD8, and therefore are considered double-positive (DP) cells, account for more than 80% of all thymocytes and are primarily detected at the corticomedullary junction of the thymus. Few DP thymocytes, which are positively selected and do not interact at high avidity with the thymic stroma, mature to become single-positive (SP) cells expressing either $CD4^+$ or $CD8^+$, which are found in the medulla. Most DP thymocytes fail selection and undergo apoptosis, which can be mediated by several mechanisms.[9] The extrinsic apoptotic pathway often involves upregulation of Fas receptor expression and stimulation through the Fas receptor, followed by activation of the caspase pathway and cleavage of proapoptotic molecules. The intrinsic apoptotic pathway can be triggered by nuclear DNA damage,[10] as well as induction of the proapoptotic molecule Bim, as recently shown in lymphocytes treated with the PNP inhibitor forodesine.[2] The intrinsic pathway might also be activated by downregulation of antiapoptotic molecules, such as Bcl2, followed by disruption of mitochondrial membrane potential (MMP) and mitochondrial damage, as shown in thymocytes from ADA-deficient mice[11] and in cells treated with forodesine.[2] Additionally, we have shown in normal thymocytes that accumulation of purine metabolites can bypass the extrinsic and nuclear apoptotic pathways, directly damaging the mitochondria and leading to dissipation of MMP, release of cytochrome c from the mitochondria to the cytosol, and cell death.[5]

Recently, OP9 bone marrow stromal cells expressing the delta-like ligand 1 (OP9-DL1 cells) were found to induce differentiation of hematopoietic stem cells (HSCs) into thymocyte-like cells *ex vivo*.[12] Avoidance of confounding factors that affect thymocytes *in vivo* and the synchronized production of large quantities of cells at similar maturation stages by the OP9-DL1 system enabled identification and analysis of several molecules and pathways important for thymocyte development.[13-15]

To better understand the effects of PNP deficiency on thymocytes, we took advantage of the accessibility to thymic tissue from many PNP-KO mice, as well as the ability to generate *ex vivo* thymocyte-like cells from HSCs of PNP-KO mice. We show that accumulation of dGuo preferentially damages the mitochondria of DP thymocytes, resulting in cell apoptosis. Moreover, restoring PNP in PNP-deficient thymocytes can avert the dGuo-induced apoptosis.

METHODS

Mice

Six-week-old PNP-KO (C57BL/6) mice and normal littermates maintained in a pathogen-free environment at the animal facility of the Hospital for Sick Children, Toronto, Canada, were used for all experiments. PNP deficiency was determined based on the results of Southern blot analysis and the absence of PNP enzyme activity in tail blood samples.[5] The institution's Animal Care Committee approved all procedures in accordance to the Canadian Council of Animal Care Guidelines.

PNP activity and PNP fused to TAT protein transduction domain

PNP activity was measured and defined as previously described.[5,16] The recombinant protein of the PNP fused to TAT protein transduction domain (TAT-PNP) was produced as previously described.[16]

Fluorescence-activated cell sorting analysis

Single-cell suspensions were stained with the appropriate antibodies or isotype-matched controls, as detailed in the Methods section in this article's Online Repository at www.jacionline.org.

DP and SP thymocyte isolation

Isolation of thymocytes was performed by means of magnetic depletion on MACS columns (Miltenyi Biotec, Auburn, Calif), as detailed in the Methods section in this article's Online Repository.

OP9-DL1 cultures

Lineage-depleted HSCs were isolated from freshly collected bone marrow by means of removal of cells expressing CD5, CD45R (B220), CD11b, GR1, 7-4, and Ter-119 with the Lineage Cell Depletion Kit (Miltenyi Biotec). Lineage-depleted HSCs (10^5 cells/well) were added to near-confluent OP9-DL1 cells (kindly provided by J. C. Zúñiga-Pflücker, Toronto, Ontario, Canada). HSCs were maintained in αMEM (Invitrogen, Burlington, Ontario), 20% characterized heat-inactivated FBS (ThermoScientific, Nepean, Ontario), 5 ng/mL fms-like tyrosine kinase-3 ligand, and 1 ng/mL IL-7 (both from R&D Systems, Minneapolis, Minn), as previously described.[12] Nonadhesive cells were transferred every 3 to 4 days to fresh OP9-DL1 cells. PNP activity was irreversibly inhibited in OP9-DL1 cells by means of treatment with 20 μmol/L forodesine (kindly provided