Purine Nucleoside Phosphorylase (PNP) activity regulates the expression of RANKL by lymphocytes: a role for the purine salvage pathway in the osteoimmune system.

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Introduction

Bone mass is maintained due to the balanced activities of bone forming cells (osteoblasts) and bone reabsorbing cells (osteoclasts). Bone marrow stromal cells and osteoblasts support osteoclastogenesis via the synthesis of receptor activator of NF-κB ligand (RANKL). RANKL binds to a cell membrane receptor (RANK) in osteoclast precursors. In the presence of macrophage colony stimulating factor (M-CSF), these events lead to differentiation of precursors to mature and active osteoclasts (Wyzga et al, 2004). Activated T cells also have been associated with increased osteoclast formation and accelerated bone resorption caused by inflammation in vivo and in vitro (Weitzmann et al. 2006).

Purine Nucleoside Phosphorylase (PNP) is a purine-metabolizing enzyme that catalyzes the reversible phosphorolysis of purine nucleosides such as deoxyinosine and deoxyguanosine (dGuo) to their respective bases and deoxyribose-1-phosphate (Bantia et al., 2004). It is a key enzyme in the purine salvage pathway of mammalian cells where inosine and guanosine derived mainly from ribonucleotides hydrolysis, and 2’-deoxyguanosine derived from DNA degradation are its main substrates (Bzowska et al. 2000). T-cells rely heavily on PNP activity to maintain its functions and are particularly sensitive to PNP deficiency, which is attributed to a relatively high level of kinase and low level of nucleotidase activity compared to other cells (Bantia et al., 2004). PNP has recently become a potential target for drug development since analogs of the PNP transition state have been synthesized and tested with promising results. Furthermore, PNP activity has been detected in
biological fluids which expand our understanding of its array of functions (Silva, et al. 2004). We have recently demonstrated that PNP activity is increased in periodontally diseased sites, and that periodontal treatment has effectively decreased PNP activity in chronic and aggressive periodontitis. Furthermore, we have shown that the pharmacological inhibition of PNP arrests bone loss induced by inflammation using an animal model. Therefore, the aim of the present set of experiments was to shed light on the mechanisms involved in the regulation of bone loss mediated by PNP.

Materials and Methods

1. In vitro model of osteoclastogenesis

Osteoclast precursor cells were obtained from bone marrow of mouse tibiae. The cells were resuspended in α-MEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were plated at a density of 5 X 10⁵ cells/well in a final volume of 0.5 ml using 48-well plate, and incubated at 37ºC in 5% CO₂ atmosphere. Osteoclast precursor cells were cultured for 7 days in the presence of RANKL (50ng/ml) and MCSF (30ng/ml). Total RNA was isolated at 0, 2, 4, and 6 days after induction of osteoclastogenesis.

2. Enrichment of T CD4 Memory cells

Monocyte fractions were obtained from human blood after purification by density gradient centrifugation. T CD4⁺ Memory cells were isolated using magnetic cell separation beadsthrough negative selection procedures. T CD4⁺ cells were plated and incubated at 37ºC in 5% CO₂ atmosphere. After 48h, all T CD4 cells were incubated with Concanavalin A (Con A) (5μg/ml). Then, cells received either with a PNP transition state analog (Immucillin H, 80nM) or PBS, and total RNA was isolated at 0, 4, 7 and 11h after T cells activation.

3. Differential expression of RANKL and PNP

cDNA was synthesized from 10ng of total RNA and used in Real-time RT-PCR performed in duplicates using primers and TaqMan probes for mouse PNP, human PNP and human RANKL. Mouse β-actin or human GAPDH were used for normalization of the PCR reaction. Relative expression was calculated according to a previously described method (2^ΔΔCt) having the control tissue as the calibrator.
**Results and Discussion**

Our results showed that the expression of PNP in precursor osteoclast cells decreased throughout the *in vitro* differentiation osteoclasts. Progressive decrease of PNP expression in osteoclasts emphasizes its role in the undifferentiated state, characterized by intense proliferation, when cells need high levels of nucleotides to sustain replication and gene expression. RANKL expression was upregulated in human memory T CD4⁺ cells, a 3-fold increase after 4h of stimulation with Con A compared with control cells. Expression at 7 and 11 hours post stimulation showed a progressive decrease, but still higher than control cells. Incubation of Con A-stimulated cells with a PNP inhibitor (Immucilin H) caused downregulation of RANKL compared with cells stimulated with Con A. We have also analyzed the expression of PNP in human memory T CD4⁺ cells stimulated with Con A; no major changes were observed for any timepoint, i.e., activation of T CD4 cells have not affected PNP mRNA levels.

**Conclusions**

The findings of this ongoing investigation herein reported shows that PNP plays an important role in the regulation of bone resorption triggered by inflammation. Our results suggest that one of the key lymphocyte-based osteoclast activation mechanisms, i.e., RANKL expression, can be downregulated when PNP is inhibited. *In vivo*, RANKL downregulation prevents activation and differentiation of osteoclasts that lead to mineralized extracellular matrix degradation.

**References**


