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Loss of intestinal alkaline phosphatase leads to distinct chronic changes in bone phenotype

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ABSTRACT

Background: The gut is becoming increasingly recognized as the source of various systemic diseases, and recently, it has been linked to bone metabolism via the so-called gut-bone axis. The microbiome and gut-derived mediators are thought to impact upon bone metabolism, and administration of probiotics has been shown to have beneficial effects in bone. The gut brush border enzyme intestinal alkaline phosphatase (IAP) plays an important role in controlling calcium absorption, inhibiting lipopolysaccharides, and other inflammatory mediators responsible for endotoxemia and appears to preserve the normal gut microbiota. Interestingly, IAP-deficient mice (*AKP3*$^{-/-}$) also display a significant decrease in fecal *Lactobacillus*, the genus shown to be beneficial to bone.

Materials and methods: IAP mRNA levels in mouse bone were measured using quantitative real-time polymerase chain reaction. Femurs of IAP-knockout (KO) and wild-type (WT) mice were analyzed by microcomputed tomography and histopathology. Serum levels of alkaline phosphatase, calcium, and phosphorus were measured. Target cell response upon exposure to serum from IAP-KO and WT mice was quantified using primary bone marrow macrophages.

Results: IAP was not significantly expressed in bones of WT or KO animals. IAP (alkaline phosphatase 3) expression in bone was vanishingly low compared to the duodenum (bone *versus* duodenum, 56.9 ± 17.7 *versus* 25,430.3 ± 10,884.5 relative expression, $P = 0.01$). Bone histology of younger IAP-KO and WT animals was indistinguishable, whereas older IAP-deficient mice showed a distinctly altered phenotype on histology and computed tomography scan. Younger KO mice did not display any abnormal levels in blood chemistry. Older IAP-KO animals showed an isolated increase in serum alkaline phosphatase levels

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reflecting an environment of active bone formation (IAP-WT *versus* IAP-KO, 80 ± 27.4 U/I *versus* 453 ± 107.5 U/I, $P = 0.004$). There was no significant difference in serum calcium or phosphorus levels between KO and WT mice. Serum from IAP-KO mice induced a significantly higher inflammatory target cell response.

Conclusions: Through its multiple functions, IAP seems to play a crucial role in connecting the gut to the bone. IAP deficiency leads to chronic changes in bone formation, most likely through dysbiosis and systemic dissemination of proinflammatory mediators.

Introduction

"All disease begins in the gut!" There is growing evidence that supports this statement by Hippocrates. Especially in the last few decades, the gut has become increasingly recognized as the source of various diseases throughout the body. For a long time, the only known connection between gut and bone was the key bone mineral calcium. Now there are an increasing number of studies suggesting additional ways in which the gut can influence bone health. Very recent advances have revealed that the microbiome and gut-derived mediators can impact upon bone metabolism via the so-called "gut-bone axis."[1] The most direct evidence to demonstrate how the gut microbiota interacts with the host to modulate bone structure comes from studies comparing the skeletal system of germ-free mice with conventionally housed animals.[2-4] Despite some controversial results, the key point of these studies is that the presence or absence of microbiota significantly altered the bone structure.

The administration of beneficial bacteria (probiotics) such as *Lactobacillus* has been shown to result in higher bone mineralization and greater bone strength.[1] Gut-derived inflammation and inflammatory mediators are thought to contribute to the gut-bone axis. The gut brush border enzyme intestinal alkaline phosphatase (IAP) is an anti-inflammatory factor that detoxifies a variety of bacterially derived proinflammatory factors such as lipopolysaccharides (LPS), CpG-DNA, and flagellin.[5] In addition, IAP promotes gut barrier function through upregulation of intestinal tight junctions and has been shown to promote growth of intestinal commensal bacteria, preserving homeostasis of the gut microbiota.[6,7] Interestingly, IAP-deficient mice ($AKP3^{-/-}$) display a significant decrease in fecal *Lactobacillus*. Furthermore, IAP appears to control intraluminal calcium concentrations. Given the functional roles of IAP in controlling gut permeability, gut-derived inflammation, microbiotal homeostasis, and calcium absorption, we hypothesized that the loss of IAP could lead to major changes in bone metabolism and remodeling.

Material and methods

Mice

Specific pathogen-free IAP-knockout (KO) ($Akp3^{-/-}$) mice [8] were obtained from the Burnham Medical Research Institute (La Jolla, CA) and bred at the Massachusetts General Hospital (MGH, Boston, MA) animal facility to create homozygous IAP-KO, heterozygous, and wild-type (WT) C57BL/6 littermates. Genotype was confirmed by polymerase chain reaction (PCR) analysis.[8] Female mice of different age groups (3, 4, 12, 21 mo) were used in this study. Animals were maintained in a specific pathogen-free environment at MGH in accordance with the guidelines of the Committee on Animals of MGH, Harvard Medical School (Boston, MA). All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee and adhered to the regulations of the Subcommittee on Research Animal Care of the MGH and the National Institutes of Health. Animals were euthanized per the guidelines of the Animal Veterinary Medical Association.

Microcomputed tomography

Microcomputed tomography (μCT) imaging of whole femurs was performed with a high-resolution benchtop imaging system (μCT 40, Scanco Medical, Brüttisellen, Switzerland). Scans were acquired with a 10 μm^3 isotropic voxel size, 70 kVp peak X-ray tube potential, 114 mA intensity, and 200 ms integration time.[9] Transverse and sagittal reconstructions of the data were created to display complete femoral architecture of WT and IAP-KO mice at 12 and 21 mo of age.

Histopathology

Tibial and femoral samples were fixed in 10% formaldehyde for 24 h and then decalcified in 15% ethylenediaminetetracetic acid for 2 wk. Processing for paraffin sections was then completed before H&E staining. Samples were analyzed by an independent pathologist blinded to animal group assignment.

Quantitative real-time polymerase chain reaction

Identical segments of the duodenum, liver, and bone were harvested. The duodenum was flushed with ice cold Phosphate-buffered saline. Bone marrow was flushed out with ice cold Phosphate-buffered saline. The tissue was then processed with TRIzol (Invitrogen) to isolate total RNA. IScript Reverse Transcription Supermix for real-time polymerase chain reaction was used for the generation of cDNA for all samples. Quantitative real-time polymerase chain reaction was performed with a Mastercycler Realplex (Eppendorf) using the iQ SYBR Green Supermix Kit. Primer sequences are available on request. For each sample, real-time PCR reactions were performed in triplicate and the average threshold cycle calculated. Target-gene mRNA expression was normalized using glyceraldehyde-3-phosphate dehydrogenase mRNA