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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

levels. Expression relative to control was calculated using the DDCt method after correcting for differences in PCR efficiencies. Average copy number of mRNA expression in control samples was set to 1.0.[6]

In vitro *cell response*

Serum was collected via heart puncture at the time of sacrifice. Primary bone marrow macrophages were extracted from 8-wk-old WT C57BL/6 mice after flushing out the bone marrow. Four hundred thousand cells were plated and incubated with Dulbecco's Modified Eagle's Medium growth media and portal or systemic serum. Total RNA was extracted using the RNeasy Plus kit (Qiagen) followed by reverse transcription using the iScript Kit (BIO-RAD) according to the manufacturer's protocol. The resulting cDNA was used as a template for quantitative real-time PCR using the iQ SYBR Green Supermix Kit.

Blood chemistry

Serum of IAP-KO and WT animals was analyzed at the MGH Center for Comparative Medicine, Boston, USA.

Results

IAP (alkaline phosphatase 3) is not expressed in the bone of WT mice

To investigate whether IAP is expressed in bone of WT mice, we measured IAP transcription levels in comparison to those in the liver and duodenum as negative and positive controls, respectively (Fig. 1). IAP transcription levels were similar in WT and IAP-KO mice (IAP-WT *versus* IAP-KO, 56.9 ± 17.7 *versus* 41.4 ± 21.3 relative expression, $P = 0.4$) and vanishingly low when compared to duodenal expression levels (bone *versus* duodenum, 56.9 ± 17.7 *versus* 25,430.3 ± 10,884.5 relative expression, $P = 0.01$).

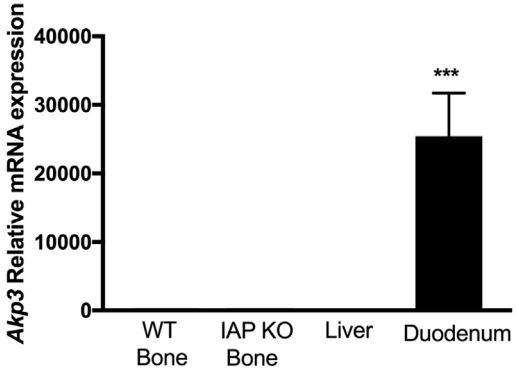

Fig. 1 – IAP (AKP3) relative mRNA expression level in the bone. IAP transcript levels in the bone of IAP-KO and WT mice compared to expression levels in the liver and duodenum as negative and positive controls. IAP transcript levels were similar to IAP-KO counterparts (IAP-WT *versus* IAP-KO, 56.9 ± 17.7 *versus* 41.4 ± 21.3 relative expression, $P = 0.4$) and vanishingly low when compared to duodenum expression levels (bone *versus* duodenum, 56.9 ± 17.7 *versus* 25,430.3 ± 10,884.5 relative expression, $P < 0.001$). Statistics: data expressed as mean ± SEM. Two-tailed unpaired Student's t test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. SEM = standard error of the mean.

Older female IAP-KO mice show distinctly altered bone phenotype on histology

Lack of IAP seems to manifest itself in a chronic process involving significant alterations in bone physiology. Histological examination revealed distinct differences between IAP-KO and WT animals (Fig. 2A-D). Notably, there was a dramatic increase in cortical thickness associated with trabecularization of the endocortical bone characterized by disruption of the cortex with islands of stromal cells and vasculature.

The consequences of IAP deficiency on bone morphology were not detectable in young animals (Fig. 2E and F).

Older female IAP-KO mice show distinctly altered bone phenotype on μCT

Compared to their WT littermates, 21-mo-old IAP-KO mice possessed a greater volume of intracortical bone with a much more disorganized and chaotic osseous structure (Fig. 3A). This IAP-KO phenotype was absent in younger mice at 12 mo of age (Fig. 3B), supporting the assumption that this process is a chronic and gradual one developing over time.

Older IAP-KO mice demonstrate an isolated increase in serum alkaline phosphatase levels but normal serum calcium and phosphorus

We measured serum concentrations of calcium, phosphorus, and alkaline phosphatase (AP) (Fig. 4A-C) in young and old IAP-KO and WT mice. Younger KO mice did not display any abnormalities after analysis of blood chemistries. Figure 4C shows the isolated increase in serum AP with advancing age in the KO animals reflecting a state of increased bone turnover, whereas calcium and phosphorus levels did not change significantly. Normal gamma-glutamyltransferase levels ruled out underlying hepatic pathology as a cause of the AP increase.

Serum from IAP-KO mice causes a significantly higher inflammatory response in target cells than serum from WT mice

To determine the extent to which systemic serum may contribute to inflammation in peripheral organs such as the bone marrow, we tested the inflammatory response of target cells exposed to systemic serum from IAP-KO and WT mice. Murine primary bone marrow macrophages were incubated with serum for 24 h followed by measurement of target cell tumour necrosis factor alpha transcription levels. Serum from IAP-KO mice induced a significantly higher target cell inflammatory response than did serum derived from WT counterparts (Fig. 5).

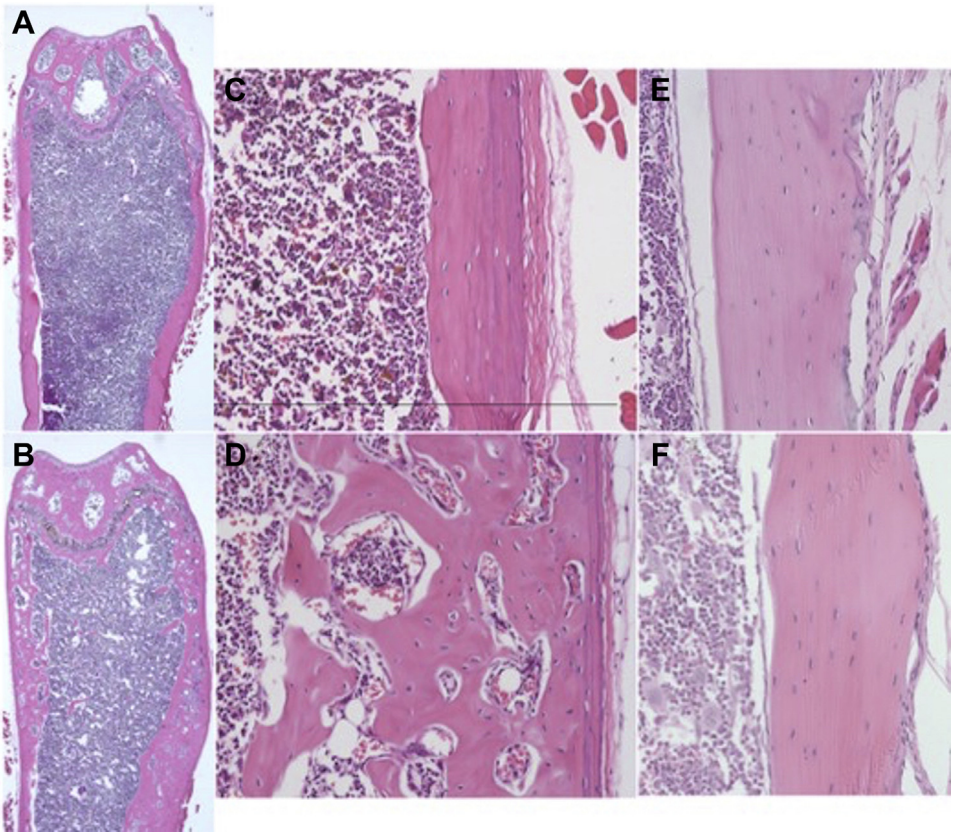

Fig. 2 – Bone histology in young and old IAP-KO and WT animals. (A and B) Low power magnification of old femurs from 21-mo-old WT (A) and IAP-KO (B) mice. (C and D) 20× magnification of WT (C) and IAP-KO (D) cortex of 21-mo-old mice. Histology examination revealed dramatic differences in the cortical bone between IAP-KO and WT animals. (E and F) Bone cortex of 12-wk-old WT (E) and IAP-KO (F) animals without any marked differences. (Color version of figure is available online.)

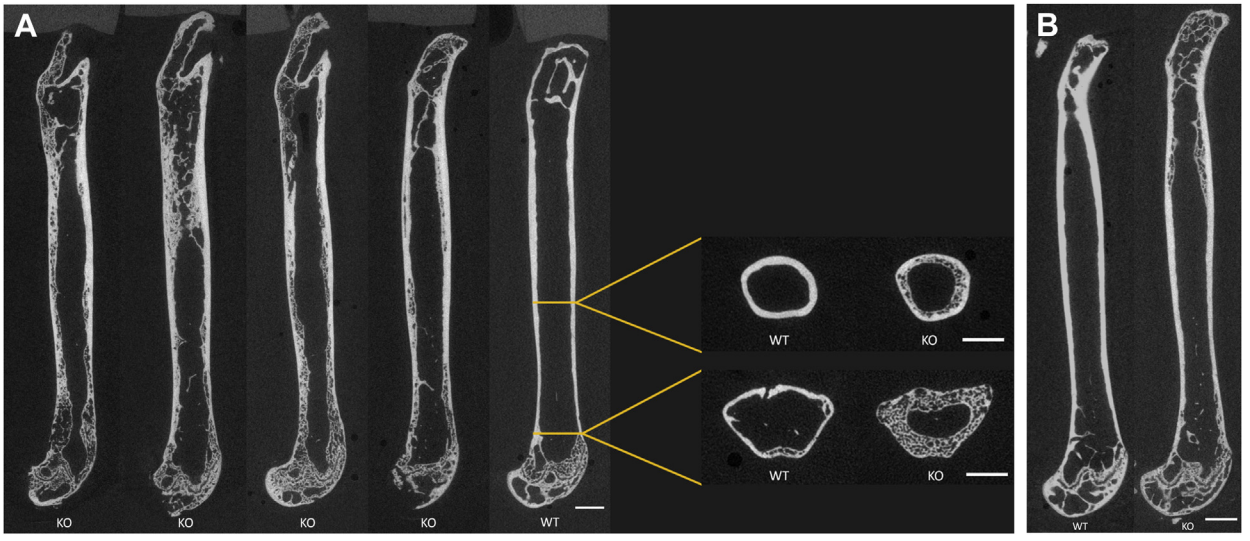

Fig. 3 – Representative sagittal and transverse μCT images of the femurs of female WT and IAP-KO mice. (A) Twenty-one months and (B) 12 mo of age. (A, right side) Transverse views of the mid-diaphysis and distal metaphysis show the distinctive skeletal phenotype that was present at 21 mo of age. Scale bars = 1 mm. (Color version of figure is available online.)

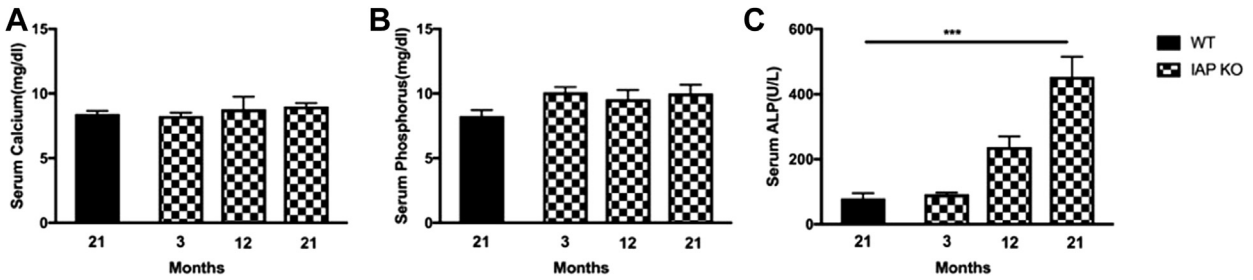

Fig. 4 – Blood chemistry levels in young and old IAP-KO and WT mice (A-C). Calcium, phosphorus, and AP in young and old IAP-KO and WT mice. Older IAP-KO animals show an isolated AP increase serum levels but normal calcium and phosphorus. Younger KO mice did not display any abnormal levels in blood chemistry analysis. Panel C shows the isolated AP increase with age in the KO animals reflecting the increased bone turnover, whereas Ca and phosphorus levels did not differ significantly. Normal μGT levels ruled out an underlying hepatic pathology causing the AP increase. Statistics: data expressed as mean ± SEM. Two-tailed unpaired Student's t test. *P < 0.05; **P < 0.01; ***P < 0.001. SEM = standard error of the mean.

Discussion

The disorganized and chaotic nature of the cortical bone observed in IAP-KO mice and thus an environment of chronic IAP deficiency suggests a pivotal role for IAP in bone metabolism. Bone is not a static structure but is constantly undergoing remodeling via osteoclast-mediated removal of mature bone and osteoblast-mediated bone formation. Remodeling enables osseous tissue respond appropriately to fractures or physical strain. An imbalance between bone resorption and formation can result in disease such as osteoporosis, arthritis, or Paget's disease. To quantify the amount of bone formation taking place, several markers can be measured in the blood. These markers are products of active osteoblasts and reflect various aspects of osteoblast function and bone formation. In our study, we used AP as a marker of active bone formation. AP is the most commonly used marker for bone metabolism, and assuming liver disease is excluded, serum levels of AP can accurately reflect the extent of new bone formation and osteoblast activity.[10]

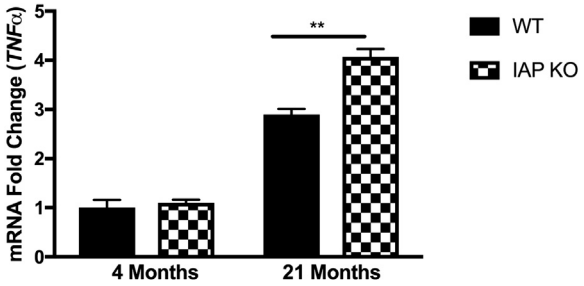

Fig. 5 – Inflammatory response of target cells after incubation with serum from IAP-KO and WT mice. There was no marked difference in the target cell response to serum from 4-mo-old IAP-KO and WT mice. Serum from 21-mo-old IAP-KO animals induced a significant higher inflammatory target cell response than serum derived from WT counterparts (P < 0.01). Statistics: data expressed as mean ± SEM. Two-tailed unpaired Student's t test. *P < 0.05; **P < 0.01; ***P < 0.00. SEM = standard error of the mean.

In our present study, serum AP levels increased concurrently with the changes observed in cortical bone on μCT and histological analysis. No increased levels of AP were detected in young animals and clearly raised but still high-normal serum AP levels were seen in 12-mo-old IAP-KO mice. Twenty-one-month-old mice exhibited significantly increased serum AP levels mirroring the altered cortical bone phenotype seen in aged IAP-deficient mice. This distinct and unique phenotype observed in older IAP-KO mice may have multiple, diverse explanations, but it clearly reveals the huge impact of intestinal alterations on the function of peripheral organs such as the bone. However, to distinguish the exact role of IAP in bone formation and bone resorption, further tests are needed.

For a long time, the only known connection between the gut and bone was the key bone mineral calcium. A study by Brun et al suggested that IAP plays a major role in the regulation of calcium absorption.[11] They showed that higher luminal calcium concentrations increase the activity of IAP bound to the brush border, which then results in a subsequent decrease in the percentage of calcium absorbed.

Furthermore, very recently, the same working group revealed a state of increased calcium uptake and improved trabecular bone structure in 4-mo-old female IAP-KO mice.[12] However, in our present study, the loss of IAP was not associated with altered serum calcium levels in young or old KO mice suggesting the involvement of environmental factors or additional mechanisms in controlling intestinal calcium absorption. In addition, the increased "trabecularization" of the cortical bone that we now show in aged IAP-KO animals emphasizes the role of IAP in bone metabolism.

Several studies provide evidence of additional ways in which the intestine can impact upon bone health. The gut microbiome seems to influence physiological bone remodeling and homeostasis, but the role of the commensal gut microbiota on physiological tissue remodeling and homeostasis at extra-intestinal sites is still largely unknown, and until now, findings have been controversial.[13] There is evidence suggesting that the intestinal microbiota interacts with the host to modulate bone density, which comes from studies comparing germ-free mice with conventionally housed animals. In one study, germ-free mice showed a clear increase in femoral trabecular bone

volume and cortical bone volume compared to conventionally housed animals.[2] The authors attributed these findings to a decrease in osteoclast numbers and reduced expression of inflammatory cytokines in the bone and bone marrow. In contrast, other studies have shown more recently that the normal gut microbiota supports skeletal growth in conventional *versus* germ-free housed mice.[3,4] The key point of these studies was that the presence or absence of microbiota significantly changed the structure of the bone. Antibiotic models provide further evidence for an association between gut microbes and bone, reporting larger bone sizes and higher bone mineral content in antibiotic-treated animals.[14] In a previous study, we demonstrated that IAP-KO mice display an overall decrease in the number of intestinal bacteria, and furthermore that oral supplementation with IAP in WT mice was able to rapidly restore the normal gut flora in mice exposed to antibiotics.[15] In this context, it is important to note that administration of beneficial bacteria or probiotics led to higher bone mineralization and greater bone strength, respectively, in preclinical models. Of note, the *Lactobacillus* genus has been particularly shown to possess these beneficial effects in bone.[1] Interestingly, in IAP-KO mice, there was a significant reduction in stool *Lactobacillus*.[15] However, the effectiveness of prebiotic or probiotic treatment seems to be dependent upon stage of development and gender.[1] We only tested the impact of IAP deficiency on the bone in female mice and thus cannot report on any gender-specific differences.

An unimpaired gut barrier is essential to prevent the entry of gut-derived, potentially harmful mediators into the portal vein, the first step of their systemic dissemination. Consequently, many disorders have been proven to be associated with not only increased gut barrier permeability, for example, intestinal diseases such as Crohn's disease and ulcerative colitis, but also a variety of other nonintestinal diseases including metabolic syndrome, osteoarthritis, and Alzheimer's disease.[16-23] The microbiome and gut-derived mediators such as LPS and flagellin are also thought to impact upon bone metabolism.[24,25] In addition, inflammation and proinflammatory cytokines appear to be an important determinant for low bone density in pediatric inflammatory bowel disease patients and are further associated with osteoporosis.[26-28] LPS and cytokine levels are increased in portal and systemic serum of IAP-KO mice (data not shown) and systemic serum from IAP-KO animals caused a significantly higher inflammatory response when incubated with primary bone marrow macrophages, which served as target cells. The present study illustrates the impact of chronic dissemination of gut-derived inflammatory mediators on peripheral organs such as the bone.

Conclusion

IAP deficiency leads to chronic changes in the bone, most likely through dysbiosis and systemic dissemination of proinflammatory mediators. Given its functional roles in the control of gut permeability, inflammation, and microbiotal homeostasis, IAP appears to play a major role in bone metabolism. The changes we have observed in IAP-KO animals throughout this study were not associated with pathologic serum calcium levels. The aged IAP-KO mouse serves as an example to illustrate the gut-bone axis. Further work is needed to elucidate the exact mechanisms behind the effect of IAP on bone metabolism.

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Authors' contribution: F.K. designed research, conducted experiments, acquired data, analyzed data, wrote the article. F.A., S.R.H., R.V., E.L., Y.L., R.S.H., J.M.R., A.R.M, and F.C.K. conducted experiments, acquired data, and analyzed data. M.A. and D.J.B. performed microcomputed tomography and analyzed data. M.L.B., M.B.D., and R.A.H. designed research, analyzed data, and revised the article critically for important intellectual content. All authors revised and approved the article for publication.

Disclosure

The authors have declared that no conflicts of interest exist.

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