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The intestinal microbiome and skeletal fitness: connecting bugs and bones

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Abstract

Recent advances have dramatically increased our understanding of how organ systems interact. This has been especially true for immunology and bone biology, where the term “osteimmunology” was coined to capture this relationship. The importance of the microbiome to the immune system has also emerged as a driver of health and disease. It makes sense therefore to ask the question: how does the intestinal microbiome influence bone biology and does dysbiosis promote bone disease? Surprisingly, few studies have analyzed this connection. A broader interpretation of this question reveals many mechanisms whereby the microbiome may affect bone cells. These include effects of the microbiome on immune cells, including myeloid progenitors and Th17 cells, as well as steroid hormones, fatty acids, serotonin and vitamin D. As mechanistic interactions of the microbiome and skeletal system are revealed within and without the immune system, novel strategies to optimize skeletal fitness may emerge.

Keywords

Microbiome; osteoblast; osteoclast; osteoimmunology

1. Introduction

Bone mass is the major determinant of fracture risk with aging and is regulated by a complex interplay of cellular, hormonal and metabolic pathways [1,2]. At a cellular level two cell types, the osteoblast and osteoclast, synthesize and degrade bone throughout life, respectively. A third cell, the osteocyte, is derived from osteoblasts and resides within the bone matrix to monitor biomechanical stress and coordinate osteoblast and osteoclast activity. Both adaptive and innate immune cells influence osteoblasts and osteoclasts through factors such as cytokines. The calcium/vitamin D/parathyroid hormone (PTH) axis is the most well known hormonal pathway. Decreases in serum calcium stimulate the release

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of PTH, which raises the serum calcium level by promoting osteoclastic bone resorption and calcium absorption in the gut while decreasing renal calcium excretion. Steroid hormones, including estrogen and glucocorticoids, also profoundly affect bone cells. Other reviews in this issue focus on the role of the microbiome on local bone diseases, such as periodontitis, rheumatoid arthritis and the spondyloarthropathies. In this review, the mechanisms by which the gut microbiome may affect *systemic* bone metabolism are considered.

Although direct data is limited, it is easy to envision how the microbiome could influence bone metabolism. Since bone cells are unlikely to come in direct contact with microbes outside of the oral cavity and deep seated infections, effects must be mediated indirectly by cells or soluble factors. The interaction of the microbiome with the skeletal system can be framed within one of three categories considered here (Figure 1). These include effects of the microbiome on 1) the immune system, also known as osteoimmunology [2], 2) hormonal pathways (e.g. steroid hormones, PTH and vitamin D), and 3) the production of bacterial metabolites that could signal to bone cells. Before addressing these potential mechanisms, an overview of papers that directly address the connection between the intestinal microbiome and skeletal biology is provided.

2. The microbiome and bone – direct evidence of interactions

How manipulations of the intestinal microbiome may affect bone mass has been examined in three contexts: following the ingestion of pre- and probiotics, after treatment with broad-spectrum antibiotics and under germ-free (GF) conditions. Here, each is reviewed. Due to limited data on this topic in humans, the discussion is largely limited to murine studies.

2.1. Prebiotics and Probiotics

Prebiotics are non-digestible food constituents like dietary fiber and oligosaccharides that modulate bacterial communities in the gut with beneficial effects on the host. Inulin, oligofructose and galactooligosaccharides are the best-studied prebiotics in terms of their effects on bone (reviewed in [3]). Abrams et al. built on earlier studies [3] to show that inulin-type fructans increased bone mineral content (BMC) and bone mineral density (BMD) in adolescents [4]. Similar results were obtained in animals treated with inulin type fructans. Prebiotics may increase calcium uptake thereby promoting bone mineralization by augmenting total body calcium [3]. Mechanistically, fermentation of these sugars into short chain fatty acids (SCFA) by the microbiota and acidification of the gut lumen enhance calcium solubility to increase absorption. Whether this is the sole pathway by which prebiotics increase bone mass is unclear.

Probiotics are microorganisms that after ingestion confer beneficial effects on the health of the host. The effect of probiotics and fermented food products on bone mass in animals has been reviewed [5]. Here, two recent papers are discussed. McCabe et al. treated male and female mice with *Lactobacillus reuteri* ATCC PTA 6475 three times per week for 4 weeks [6]. This strain was chosen because it suppresses tumor necrosis factor (TNF) production in monocytes through histamine [7]. Given the effect of inflammatory cytokines, like TNF, on promoting osteoclast activity and inhibiting osteoblasts [2], the authors reasoned that modulation of inflammation by *L. reuteri* 6475 may increase bone mass. Gavage with this

probiotic reduced intestinal *Tnf* transcripts and increased trabecular bone mass in male but not female mice. The increase in bone mass was associated with elevated bone formation rates, without changes in a serologic biomarker of osteoclast activity [6]. Similar experiments should be done with an *L. reuteri* 6475 mutant incapable of generating histamine [7] to determine whether this pathway indeed mediates its positive effects on bone formation.

The lack of an effect of *L. reuteri* 6475 in female mice prompted the investigators to examine this probiotic in the ovariectomy model of post-menopausal osteoporosis [8]. One week after ovariectomy, mice received *L. reuteri* 6475 thrice weekly for 4 weeks. This treatment protected mice from trabecular bone loss and was associated with reduced levels of *Tnfsf11* (Receptor activator of NF- κ B ligand (RANKL)) and *Acp5* (TRAP5b; a marker of osteoclast number) transcripts in whole bone mRNA. *L. reuteri* 6475 induced significant changes in bacterial diversity with an increase in *Clostridiales* and a decrease in *Bacteriodes* species. Bone marrow (BM) from mice treated with *L. reuteri* 6475 contained fewer CD4⁺ T-cells and generated fewer osteoclasts when cultured *ex vivo* with RANKL. It remains unclear whether *L. reuteri* 6475 prevents bone loss after ovariectomy by influencing osteoclasts or osteoblasts, or both. Taken together, these data support the notion that bacterial communities in the gut influence bone metabolism.

2.2 Effects of antibiotics on bone

Antibiotics are widely used to combat infection and promote livestock growth. Although these drugs change the microbial community structure, only recently have their effects on metabolism been examined [9,10]. With respect to bone metabolism, two papers provide a glimpse of the effect antibiotics have on the skeleton. Cho et al. treated mice at the time of weaning with a variety of antibiotics in their drinking water, including penicillin, vancomycin, penicillin plus vancomycin or chlortetracycline [11]. In each case a sub-therapeutic dose was used, which altered the composition of the microbiome, but not the overall bacterial census. Compared to mice drinking antibiotic-free water, mice on each regimen displayed an increase in BMD 3 weeks after antibiotic initiation. The difference in BMD was no longer evident 7 weeks after antibiotics. The mechanism by which sub-therapeutic antibiotics resulted in short term changes in bone mass was not determined. In addition, the experimental design did not exclude the possibility that these drugs caused changes in bone mass independently of their antimicrobial activity. However, similar effects were observed with different antibiotics, each with a varied structure and mechanism of action, suggesting that changes to the microbiome were the common denominator and the cause of the bone mass increase. The second study exposed mice to low dose penicillin either from before birth (treating the pregnant dam) or from weaning through 20 weeks of age [12]. Female mice displayed a small but statistically significant increase in BMC and BMD. In contrast, BMC was reduced in male mice. More studies are needed to define the antibiotic regimens that influence bone mass. Given the widespread use of antibiotics, it will be important to ascertain their effects on bone beyond the laboratory setting.

2.3 Bone parameters in GF animals

Surprisingly, the relationship of the GF state on bone mass has received little attention. Sjogren et al. found a 40% increase in trabecular bone in 7-week-old GF female C57/BL6 mice compared to age- and sex-matched mice raised under conventional conditions [13]. GF mice had normal bone formation rates, but a reduction in the fraction of the bone surface covered by osteoclasts, suggesting that the increase in bone mass was due to reduced bone resorption. Importantly, the authors showed that colonization of GF mice with gut microbiota at 3 weeks of age normalized BMD by 7 weeks of age. The authors made three other noteworthy observations. First, the BM of GF animals had fewer osteoclast precursors and generated fewer osteoclasts when cultured *ex vivo* with RANKL. Second, GF animals had lower numbers of T cells in their BM. Lastly, lower expression levels of pro-osteoclastogenic cytokines such as *IL6* and *Tnf* were found in bones from GF mice. This important publication showed that GF animals manifest increased bone mass, which is reversible upon colonization with a normal gut flora. It suggests further that reduced numbers of T cells, osteoclast precursors or cytokines may drive the bone phenotype in GF mice.

Periodontal disease is a chronic inflammatory state, which causes local bone erosion and tooth loss. This topic has been reviewed elsewhere [14,15]. Recent data suggests that oral commensals in the absence of periodontal disease regulate the amount of alveolar bone, the maxillary and mandibular ridge in which teeth reside. Hajishengallis et al showed that mice living under specific pathogen free (SPF) conditions lose alveolar bone with age, while those in a GF environment accrue bone [16]. When GF and SPF mice were co-housed, the commensal oral microbiota was transmitted within 2 weeks and the amount of alveolar bone in the GF animals decreased to the level found in the SPF animals by 16 weeks. The SPF state was associated with significantly higher mRNA transcript levels of pro-inflammatory cytokines and chemokines, as well as *Rankl*. Irie et al. performed a more detailed analysis of the alveolar bone in GF versus SPF mice. They identified an increase in osteoclast numbers and RANKL-positive cells on the alveolar bone juxtaposing the teeth in SPF mice [17]. In addition, the epithelium lining the gingival sulcus of SPF mice contained more neutrophils, T-cells and IL-17 expressing cells. Thus, physiologic regulation of alveolar bone involved the microbiota.

3. Microbiota and osteoimmunology

3.1 Myeloid ontogeny and the microbiome

Osteoclasts develop from myeloid precursor cells under the influence of the cytokine RANKL. Thus, alterations in myelopoiesis by the microbiota leading to changes in the number of osteoclast precursors or their capacity for differentiation could influence bone mass (Figure 2). The report of elevated bone mass in GF mice by Sjogren et al. used flow cytometry and osteoclast culture assays to demonstrate that these animals had fewer BM osteoclast precursors than controls [13]. The flow cytometric definition of an osteoclast precursor used in this publication ($CD11b^{+}GR1^{-}$) however was inconsistent with other studies that more narrowly defined osteoclast precursor in murine BM [18,19]. For example, we identified a small BM myeloid population (<2% of BM cells) containing the majority of

osteoclast precursor activity as being CD11b^{low/-}Ly6C^{hi}Ly6G^{neg} [18]. Since the anti-GR1 antibody binds both Ly6C and Ly6G, osteoclast precursors are GR1⁺. In addition, intrinsic differences in the capacity of osteoclast precursors from GF mice to differentiate in vitro may explain the culture results reported in Sjogren et al [13]. Despite these issues, emerging data supports the notion that intestinal microbiota broadly regulate myeloid cell development.

Khosravi et al. showed that GF mice have fewer neutrophils (Gr1^{hi}CD115^{neg}) and monocytes (Gr1^{hi}CD115^{hi}) in their BM [20]. This defect mapped to a reduction in granulocyte-monocyte progenitors (GMP) downstream of pluripotent Lin⁻ Sca1⁺ c-kit⁺ cells (LSK, hematopoietic stem cells (HSC)). The reduction in BM monocytes was reversed by colonization or by adding heat killed bacteria to drinking water as a source of microbial associated molecular patterns (MAMPs). Similarly, Balmer et al. found lower numbers of CD11b⁺Ly6C⁺ monocytes and CD11b⁺Ly6G⁺ granulocytes in the BM of GF mice compared to SPF mice [21]. Again, GF mice had fewer GMPs and colonization increased myeloid cell numbers. Interestingly, myelopoiesis in wild-type GF animals could be restored by injection of serum from SPF mice. This response required the adapter molecules MyD88 and TICAM1, which mediate Toll-like Receptor (TLR) signaling, and was unaffected by heat-inactivating the serum, suggesting that circulating MAMPs in the serum of SPF animals promote myelopoiesis. These reports suggest that MAMPs derived from gut commensals might also influence cells of the osteoclast progenitor lineage [20,21]. Whether MAMPs are liberated at a systemic level to affect the BM compartment directly, or stimulate production of humoral factors in other organs, which affects myeloid cell differentiation by diffusing to the BM to needs to be resolved.

3.2 CD4⁺ T cell subsets and bone

In a seminal paper in 1999, Kong et al. described that activated T cells regulate bone loss by stimulating osteoclastogenesis [22]. This effect was mediated by RANKL and provided a potential explanation for the local bone destruction in RA and the generalized osteoporosis associated with this, and other, systemic inflammatory diseases [23]. Studies since have identified additional pathways for the control of osteoclast development by T cells (Figure 2). Li et al. found that CD40L-CD40 signaling between T and B cells facilitated the secretion of osteoprotegerin (OPG), an endogenous RANKL inhibitor, by B cells [24,25]. B cell deficient mice as well as *Cd40*^{-/-} and *Cd40L*^{-/-} mice were found to be osteoporotic [24] suggesting that this pathway is physiologically important. There is also experimental evidence to suggest that T cells mediate bone loss associated with estrogen withdrawal and hyperparathyroidism (reviewed in [2,26]). The effect of T cell-derived cytokines on bone cells is well studied [27]. For example, interferon- γ (IFN γ) and IL-4, the signature Th1 and Th2 cytokines, respectively, inhibit osteoclastogenesis [28,29]. IL-17A, the defining cytokine of Th17 cells, stimulates osteoclast formation directly [30] and indirectly by promoting RANKL expression in stromal cells [27]. Moreover, Th17 cells express higher RANKL than other Th subsets suggesting they may directly promote osteoclastogenesis [31]. In contrast, regulatory T cells inhibit osteoclastogenesis via a CTLA-4 pathway [32-34]. The effect of T cell derived cytokines on osteoblasts is less well studied, but they can decrease or increase osteoblast activity depending on the disease context [2,35].

Recent studies have started to reveal a close relationship between the gut microbiota and the differentiation of T helper cells and regulatory T cells (Treg). Ivanov et al. identified segmented filamentous bacteria, an intestinal commensal inhabitant in some mouse colonies, as the driver of Th17 helper cell differentiation [36,37]. Importantly, SFB affect immune responses outside the gut. SFB colonization biases the immune system toward a Th17 phenotype, driving the development of autoimmune arthritis and experimental encephalomyelitis [38,39]. In contrast, Atarashi et al. reported that clostridia species impact the development of Treg cells. Colonization of GF mice with clostridia normalized the number of Treg cells in the colon, lung, liver, and spleen of these mice [40]. Although these colonization studies did not analyze bone parameters, the available evidence suggests that the intestinal microbiota profoundly affect T cell differentiation and that T cells exert important effects on bone cells. Thus, it is likely that dysbiotic states could negatively impact bone health through T cell mechanisms (Figure 2).

4. Microbiota and metabolic bone pathways

4.1 Calcium absorption and vitamin D

Although genetics are the major determinant of peak bone mass [41], nutrition modulates bone accrual as well as post-menopausal bone loss. Dietary calcium in particular has a positive effect on bone mass throughout the life although the benefit is modest [42,43]. Absorption of dietary calcium occurs in the small intestine through active transport via epithelial calcium channels and by passive transport throughout the gut. As mentioned in section 2.1, calcium absorption may be influenced by non-digestible polysaccharide prebiotics such as inulin, which increase calcium solubility through SCFA and changes to luminal pH mediated by the microbiota [3]. Vitamin D is an essential regulator of intestinal calcium absorption. While data as to whether gut microbiota affect vitamin D metabolism are lacking, the opposite appears to be the case. Ooi et al. showed that *Cyp27b1*^{-/-} mice which cannot generate 1,25(OH)₂ vitamin D (the active form of vitamin D) develop more severe colitis than wild-type controls when exposed to dextran sulfate sodium. This effect was ameliorated by either 1,25(OH)₂ vitamin D supplementation or antibiotic treatment, suggesting that vitamin D deficiency causes intestinal dysbiosis and predisposes to intestinal inflammation [44]. Future work is needed to fully resolve this mechanism, although a growing body of literature suggests that vitamin D has anti-inflammatory effects and promotes regulatory T-cell function [45].

4.2 Fatty acids and bone cells

Prebiotics may affect bone structure through the microbiota by increasing calcium absorption, as discussed in sections 2.1 and 4.1. In addition, microbes generate fatty acid metabolites from prebiotics that regulate inflammation and possibly exert direct effects on bone cells. SCFA modulate the host in several ways: they inhibit histone-deacetylases (HDAC), activate specific G-protein coupled receptors (GPR), and induce autophagy [46]. Direct effects of the SCFA butyrate on bone cells *in vitro* have been reported including both the inhibition of osteoclast formation and either the activation or inhibition of osteoblastic cells [47,48]. Whether the inhibitory effects of sodium butyrate on osteoclasts are mediated by HDAC inhibition [49] or via one of the SCFA GPRs is unclear [50]. Moreover, SCFA

have been shown to regulate numbers and function of Treg cells in the colon [51] and thus may affect osteoclastogenesis indirectly via effects on T cells. In contrast to SCFAs, the effect of medium/long chain fatty acids on osteoclasts is likely mediated by GPR40. A GPR40 agonist suppressed osteoclast formation, decreased osteoclast precursor viability and protected mice from ovariectomy induced bone loss [50,52,53]. Consistent with this, *Gpr40*^{-/-} mice have slightly reduced bone mass [52]. In this study, the effect of GPR40 deletion on osteoblastic cells was not examined. Additionally, direct effects on bone via metabolic changes in *Gpr40*^{-/-} mice, which have alterations in insulin secretion [54], are possible. It remains to be determined whether fatty acids derived from the gut microbiota and their GPRs modulate bone structure via direct actions on osteoclasts and osteoblasts.

4.3 Microbiota, hormones and bone

The intestinal microbiome has been recognized as a virtual “endocrine organ” for its effects on the synthesis of cortisol, gastrointestinal hormones and neurotransmitters [55]. Development of the hypothalamic-pituitary-adrenal axis requires microbiota-host interactions, as *Tlr4*^{-/-} and GF animals demonstrate abnormal cortisol release in response to stress, with an exaggerated release in GF mice [56-59]. Cortisol and exogenous glucocorticoids have a plethora of negative effects on bone; they decrease calcium absorption, promote osteocyte and osteoblast apoptosis, inhibit osteoblast proliferation, and promote osteoclast survival and resorption [60]. Thus, the gut microbiota, or dysbiotic states, may affect bone via perturbation of the cortisol pathway.

Estrogen and androgens are also critical for skeletal homeostasis. Deficiency of these sex hormones increases osteoclast and osteoblast activity with a greater effect on the former, leading to net bone loss [61]. Recently, Markle et al. colonized female non-obese diabetic mice with feces from male mice. This increased circulating testosterone levels and delayed the onset of diabetes in an androgen receptor dependent manner [62]. These data suggest that intestinal microbiota could influence bone mass via sex steroids levels.

The intestinal microbiota influences systemic levels of the neurotransmitter serotonin, both via direct production of serotonin by several microbial species, as well as by regulating the availability of its precursor, tryptophan [55]. Both peripheral and central serotonin have been implicated in the control of bone mass. The major site of peripheral serotonin synthesis is the gut, mediated by the tryptophan hydroxylase TPH1. Gut specific *Tph1* deletion in mice decreased serotonin levels, increased bone formation and protected against ovariectomy induced bone loss. The suppressive effect of serotonin on osteoblasts appears to be direct, as mice with an osteoblast-specific deletion of the serotonin receptor *Htr1b* also have elevated bone mass [63].

The intestinal microbiota might also influence bone mass by regulating central serotonin levels. GF mice have increased levels of plasma tryptophan, hippocampal serotonin and serotonin metabolites compared to conventionally raised controls [59]. Interestingly, mice deficient in TPH2, the enzyme that generates central serotonin, have increased bone resorption and decreased bone formation leading to low bone mass [64]. Thus, if the gut microbiota substantively reduces central serotonin this could result in low bone mass.

5. Conclusions

The increased bone mass in GF mice, and its reversal upon colonization, strongly suggests that the intestinal microbiota significantly impact skeletal fitness. Immunomodulation is perhaps the leading candidate mechanism to explain this relationship between the microbiome and bone. However, numerous other mechanisms exist by which the microbiota may influence bone mass, including effects on hormones, mineral absorption, and via metabolites that may directly regulate bone cells. Thus the connection between bugs and bones is likely to be complex. As therapies emerge seeking to improve health by shaping intestinal microbial communities it will be important to assess whether they have untoward effects on bone or, more importantly, whether they can be leveraged to treat metabolic skeletal disease such as osteoporosis.

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Highlights

- Changing the gut microbiome in mice alters bone mass.
- The gut microbiome influences multiple pathways known to regulate bone cells.
- Gut microbes may regulate bone cells via T cells, myeloid cells and cytokines.
- Other mechanisms include steroid hormones and microbial metabolites.
- Manipulation of the microbiome could be exploited to treat bone diseases.

Figure 1. A conceptual framework to understand how the intestinal microbiota may regulate bone metabolism

Environmental exposures, antibiotics, and pre- and probiotics influence the composition of the intestinal microbiome. Microbes may change the relative activities of osteoclasts and osteoblasts through effects on the immune system and host metabolic pathways, as well as through the production of metabolites. The immune system and metabolic pathways may also influence the composition of the microbiome.

Figure 2. Osteoclastic bone resorption and the intestinal microbiome

Under conditions of normobiosis, anti-osteoclastogenic Th1, Th2 and Treg subsets balance pro-osteoclastogenic pathways mediated by inflammatory cytokines, Th17 cells and the availability of osteoclast precursors. In dysbiotic states, intestinal microbes may promote osteoclast-mediated bone loss through multiple mechanisms including 1) inflammatory cytokines, which activate RANKL expression on stromal cells and may increase osteoclast precursor numbers, 2) favoring the differentiation of Th17 cells, which directly produce RANKL and increase RANKL expression on stromal cells, and 3) suppressing the differentiation anti-osteoclastogenic Th subsets.