MINIREVIEW

Omega-3 Polyunsaturated Fatty Acids and Skeletal Health[1]

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This minireview on skeletal biology describes the actions of prostaglandins and cytokines involved in the local regulation of bone metabolism, it documents the role of lipids in bone biology, and it presents relationships between fatty acids and other factors that impact skeletal metabolism. The data presented herein show consistent and reproducible beneficial effects of omega-3 (n-3) fatty acids on bone metabolism and bone/joint diseases. Polyunsaturated fatty acids modulate eicosanoid biosynthesis in numerous tissues and cell types, alter signal transduction, and influence gene expression. These effects have not been explored in the skeletal system. Future research on n-3 fatty acids in bone biology should focus on the following two aspects. First, the further elucidation of how n-3 fatty acids alter biochemical and molecular processes involved in bone modeling and bone cell differentiation, and second, the evaluation of the potential pharmaceutical applications of these nutraceutical fatty acids in maintaining bone mineral status and controlling inflammatory bone/joint diseases.

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Dietary fat intake has remained a central focus of nutrition research in recent years and dietary guidelines for fat consumption continue to be updated in response to new information gained through epidemiological, clinical, and animal investigations (1). Links to cardiovascular disease (CVD), degenerative and inflammatory arthritis, cancer, and osteoporosis, and the recognition of fats and their derivatives as biological effectors of human pathologies serve to drive research on dietary lipids. The association of cholesterol and saturated fat with increased risk of CVD initially spurred dietary recommendations to reduce the intake of animal fat and to increase the intake of plant oils. The dramatic change in food formulations has led to a greater dietary intake of plant oils such as corn, safflower, and soybean oil, which are high in linoleic acid, and resulted in a elevated ratio of omega-6/omega-3 (n-6/n-3) fatty acids during the 20th century (2). Evidence suggests that the high intake of n-6 with an inadequate amount of n-3 fatty acids in the diet contributes to the development of certain cancers and some chronic diseases, including those of the skeletal system (bone/joint diseases).

Bone Modeling and Remodeling

Bone is a multifunctional organ that consists of a structural framework of mineralized matrix and contains heterogeneous populations of chondrocytes, osteoblasts, osteocytes, osteoclasts, endothelial cells, monocytes, macrophages, lymphocytes, and hemopoietic cells. This milieu of cells produces a variety of biological regulators that control local bone metabolism. Systemic calcitropic hormones (parathyroid hormones [PTH], estrogen, and 1,25-$[OH]_2$ vitamin D_3) and autocrine and paracrine factors, including prostaglandins, cytokines, and growth factors orchestrate the cellular activities of bone modeling to increase the length and diameter, and properly shape long bones in children. Bone grows in size and shape through the collective

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activities of cells that produce, mineralize, and resorb bone matrix. Osteoblasts produce and mineralize bone matrix, while the specialized multinucleated cells called osteoclasts cause bone resorption (3). The combined and cooperative activities of osteoblasts and osteoclasts result in a bone architecture that provides mechanical support and protection for the body. In addition, bone serves as a vital reservoir of minerals, principally calcium and phosphorus, necessary for maintaining normal cellular, neurologic, and vascular activities of the body.

Long bone growth is regulated by complex interactions between an individual's genetic potential, environmental influences, and nutrition. Long bones of children increase in length and diameter by a process called modeling. Bone modeling represents an adaptive process of generalized and continuous growth and reshaping of bone governed by the activities of osteoblasts and osteoclasts until the adult bone structure is attained. This growth requires that bone cells function normally and that adequate nutrition is provided. Bone modeling is distinct from bone remodeling in that the latter describes the local, coupled process of bone resorption and formation that maintains skeletal mass and morphology in the adult. The numerous cell-derived growth regulatory factors present within skeletal tissues exert local controls on skeletal metabolism. The prostaglandins are major players in bone metabolism, as well as in bone/joint diseases. Many of the skeletal pathologies that afflict the adult, e.g., osteoporosis and rheumatoid arthritis, are the consequence of either abnormal bone remodeling and metabolism or an inflammatory process. Recent studies suggest that the onset and severity of some of these pathologies may be delayed and lessened if bone modeling is optimized early in life or if diets are supplemented with nutrients that reduce tissue concentrations of factors that undermine skeletal health.

Osteoblasts are mononucleated bone-forming cells that originate locally from mesenchymal stem cells. Osteoblasts are recruited to a site of bone formation where they actively synthesize and secrete an organic bone matrix called "osteoid," which is composed mainly of type I collagen and other noncollagenous proteins. Following its formation, osteoid normally undergoes rapid mineralization with hydroxyapatite. In addition to synthesizing bone matrix, osteoblasts maintain a high alkaline phosphatase activity and produce numerous regulatory factors, including prostaglandins, cytokines, and growth factors. These locally produced agents are reported to stimulate bone formation or bone resorption (4–7).

Osteoclasts are large multinucleated bone-resorbing cells. They are formed at skeletal sites from the fusion of mononuclear hemopoietic precursors that arrive via the vasculature. Active osteoclasts are in contact with mineralized surfaces and produce distinctive resorptive cavities called Howship's lacunae. During bone resorption, osteoclasts produce and release lysosomal enzymes, hydrogen protons, and free radicals into a confined space next to bone that dissolve the mineral and degrade bone matrix (8).

Regulation of Bone Metabolism

Bone formation and bone resorption are regulated by systemic hormones and local factors produced in bone (4–6, 9). Systemic hormones that sustain bone formation include insulin, growth hormone (10), and estrogen (11), while those that induce bone resorption include 1,25-$(OH)_2$ vitamin D_3 (12), PTH (13), thyroid hormone (14), and glucocorticoids (15, 16). In addition, calcitonin inhibits bone resorption (17). Even though several localized compounds act on bone cells, the prostaglandins (PG) seem to be the principle mediators of bone cell function since their biosynthesis and release from bone cells and associated tissues can be induced by several cytokines as well as systemic factors. PGE_2, which is synthesized from arachidonic acid, is a potent stimulator of bone resorption and, to date, is the primary PG affecting bone metabolism. The PGs also influence the production and action of insulin-like growth factors (IGFs), which are major bone-derived growth factors (4). Once secreted and deposited in bone matrix, the IGFs are released during osteoclastic bone resorptive activity and could act in an autocrine or paracrine fashion to cause new bone formation and matrix production (18). Thus, IGFs, in concert with other bone growth factors, play an essential role in the coupling of bone formation to bone resorption. The relationship between PGs and IGFs is important in the maintenance of skeletal mass during aging, as well as being vital in optimizing acquisition of bone mass during critical stages of skeletal growth and development.

Cytokines, Growth Factors, and Osteoclastogenesis. Cytokines are extracellular signaling proteins that act on nearby target cells at low concentrations in an autocrine or paracrine fashion in cell-to-cell communications. One of the principle cellular responses produced by the action of cytokines is the synthesis and release of PGE_2.

The cytokines and growth factors involved in bone modeling and remodeling include epidermal growth factor (EGF), fibroblast growth factors (bFGF, FGF-1, and FGF-2), interferon-γ (IFN-γ), interleukins (IL-1, IL-4, IL-6, IL-10, and IL-11), platelet-derived growth factor (PDGF), transforming growth factors (TGF-α and TGF-β), tumor necrosis factor-α (TNF-α), insulin-like growth factors (IGF-I and IGF-II), osteoclast differentiation factor (ODF, also known as OPGL [osteoprotegerin ligand], RANKL [receptor activator of NFκB ligand], and TRANCE [TNF-related activation-induced cytokine]), and macrophage colony-stimulating factor (M-CSF). Most of these, such as IL-1 (19), IL-4 (20), IL-6 (21, 22), IL-11 (23, 24), TNF (9, 25, 26), EGF (27), bFGF (28), FGF-2 (29), PDGF (30), and M-CSF (31) stimulate bone resorption. Some, such as IGF-I and IGF-II (7, 30, 32, 33), FGF-2 (34), and TGF-(3 (35, 36) enhance bone formation, while others (PDGF and TGF-β) also stimulate proliferation and differentiation of collagen-synthesizing cells. Though most of the members of the interleukin family promote bone resorption, IL-10 was reported to inhibit osteoclast formation by abolishing the dif-

ferentiation of precursor cells demonstrated with a coculture system of mouse bone marrow cells and primary osteoblastic cells (37).

The recent discovery of ODF, a membrane bound/soluble protein produced by osteoblastic and stromal cells that stimulates osteoclastogenesis, and osteoprotegerin (OPG, also known as OCIF [osteoclastogenesis inhibitory factor]), a soluble ODF receptor that binds with ODF and blocks its effect on osteoclast formation, impart greater insight in the mechanisms of osteoclast differentiation and function (38). ODF appears to be a common mediator of osteoclast formation induced by many osteotropic hormones and cytokines such as PTH (39, 40), glucocorticoids (16), TNF-α, IL-1 (41), IL-6 (42), and IL-11 (43). OPG, a decoy receptor for ODF, works reciprocally with ODF to regulate the induction of mature osteoclastic cells by various factors.

It is believed that the ratio of ODF/OPG may be a regulatory mechanism controlling bone resorption. For example, PTH upregulates the expression of ODF, but downregulates OPG mRNA expression in mouse bone cells and a mouse stromal cell line ST2, which results in the stimulation of osteoclast formation (40). Hofbauer and colleagues (16) showed that glucocorticoids promote osteoclastogenesis by inhibiting OPG and concurrently stimulating ODF production by osteoblastic lineage cells, resulting in enhanced bone resorption. ODF has also been found to be involved in inflammatory and degenerative bone/joint diseases. ODF expressed on synovial fibroblasts is involved in rheumatoid bone destruction by inducing osteoclastogenesis (44). ODF is expressed by both synovial fibroblasts and by activated T lymphocytes derived from synovial tissues from patients with rheumatoid arthritis (RA), but not from normal synovial tissues. These synovial cells may contribute directly to the expansion of osteoclast precursors and to the formation and activation of osteoclasts at sites of bone erosion in RA (45). Evidence also suggests that the stimulatory effect of PGE_2 on osteoclastogenesis may be mediated by inhibition of OPG expression in bone cells. PGE_2 downregulated OPG mRNA level in human bone marrow stromal cells (hBMSC) (46) and PGE_2 decreased OPG expression in mouse calvarial osteoblasts that supported an increase in osteoclastogenesis (47).

Nagai *et al.* (48) showed that ODF participates in enhanced bone resorption in humoral hypercalcemia of malignancy. When cultured in media conditioned by SCC-4 and T3M-1 CI.2 cells (carcinoma squamous cell lines), HL60 human promyeloblastic leukemia cells differentiated into osteoclastic-like cells. The anti-ODF antibody inhibited differentiation of HL60 cells, indicating that ODF contributes to the induction of the osteoclastic phenotype observed in these cells.

IGFs. The IGFs are paracrine or autocrine regulatory polypeptides of cells. IGFs are mitogenic and stimulate differentiation in a variety of cell types. Pituitary growth hormone (GH) controls tissue biosynthesis and secretion of IGF-I postnatally and it is through IGF-I that the tissue effects of GH are mediated. Liver under the influence of GH maintains serum concentrations of IGF-I. Much of the circulating IGF is bound to plasma IGF-binding proteins (IGFBP).

Both IGF-I and IGF-II are conserved in skeletal tissue of vertebrates, including the chicken, human, and rat (13, 32, 49). The amount of IGF-I and IGF-II produced by bone cells is species dependent. In the human, neonatal mouse, and chicken, more IGF-II than IGF-I is produced in the skeletal tissues, but the adult mouse and rat have more IGF-I than IGF-II in skeletal extracts (49). While IGF-II is generally more abundant than IGF-I (50), IGF-I appears to be under greater regulatory control in bone (17, 51). For example, PGE_2 (0.01–1 μM) elevated IGF-I mRNA and polypeptide levels by 1.9- to 4.7-fold; however, PGE_2 did not increase IGF-II mRNA or polypeptide levels in bone organ cultures of fetal rat calvaria (51).

IGF-I plays an important role in maintaining bone mass. Serum IGF-I and IGFBP-3 were shown to correlate positively with bone mineral density (BMD) of the lumber spine after age, body mass index, and menopause duration were taken into account for the analysis (52). In healthy postmenopausal women, Garnero (53) reported that decreased serum concentrations of IGF-I were strongly associated with an increased risk of osteoporotic fractures, independent of BMD. It is well recognized that IGF-I level in circulation decreases with age. The reduction of the anabolic process mediated by IGF-I may account for the slow and progressive loss of bone mass that takes place after menopause (54).

A lower IGF-I level was associated with lower bone and lean body mass in hip fracture patients than in an age-matched group (55). Low BMD is one of the most significant of many factors contributing to the incidence of hip fractures. Resistance training in older women with low bone density resulted in increased IGF-I level and greater strength gain (56). The IGF-I level of these women was significantly lower than an age-matched healthy normal group before training.

Eicosanoids: Proper Balance for Optimal Skeletal Health. Certain eicosanoids (prostaglandins and leukotrienes) also exert stimulatory effects on bone formation and resorption. In 1970, Klein and Raisz (57) reported that PGE_1, PGE_2, PGA_1, and $PGF_{1\alpha}$ increased the release of ^{45}Ca into the media from cultured fetal rat bone. Since then, numerous studies have demonstrated that PGE stimulates bone formation as well as bone resorption (6, 58, 59). For example, PGE_2 stimulated collagen synthesis in cultured rat calvariae (60) and osteoblastic cells (61), and also increased the proliferation of osteoblasts in culture (62). PGE_2 was reported to increase cortical bone mass and intracortical bone remodeling in both intact and ovariectomized rats (63), and increased proximal tibial metaphyseal bone area in osteopenic ovariectomized rats (64). PGE_2 at 3 mg/kg body wt per day increased tibial shaft cortical bone formation rate by enhancing modeling activity in aged male rats (65).

On the other hand, PGEs also stimulate bone resorption. Raisz *et al.* (6) reported that infusion of PGE_2 at a high concentration depressed osteogenesis in fetal rat calvariae. Human subjects given PGE_1 treatment (10 μg of lipo-PGE_1 intravenously daily for 14 d or twice a week for 7 weeks) did not demonstrate an increase in bone formation, but showed an increase in bone resorption (66). PGE_2 has been shown to mediate the effects of 1,25-$(OH)_2$vitamin D_3 (67), cytokines (TNF-α [68] and IL-3 [67]), and growth factors (TGF-β [69], PDGF [70], and bFGF [71]) in enhancing bone resorption. Elevated production of PGE_2 has been associated with several osteolytic disorders in humans, including bone loss associated with dental cysts, failing joint prostheses, chronic osteomyelitis, and certain neoplasms of bone (58). The stimulatory effect of rhBMP-2 on osteoblast (isolated from human periodontal ligament) differentiation was modulated by local PGE_2 levels; that is, at lower levels (10^{-10} to 10^{-8} M) PGE_2 enhanced ALPase activity in cell culture and at a higher concentration (10^{-6} M), suppressed osteoblast differentiation (72). Thus, PGE_2 effects on bone formation in animal models seems to be dose related—stimulatory at low concentrations and inhibitory at high concentrations (60, 73, 74).

PGE_2 is an important factor in mediating the effects of several osteolytic cytokines such as IL-1, IL-6, and TNF-α, and calcitropic hormones. PGE_2 stimulates the release of IL-6, an essential factor in osteoclastogenesis, from various mesenchymal cell lines (MC3T3-E1, ST2, UMR-106, and fibroblastic L929) (75). In human osteoblast (MG-63 cells) cell culture, anti-PGE_2 antibody markedly reduced the IL-1β-stimulated production of IL-6 (76). In the same study, IL-1β also induced cyclooxygenase-2 (COX-2) expression, PGE_2 production, and EP_1 receptor signaling prior to IL-6 production. NS-398, a selective COX-2 inhibitor, suppressed the production of PGE_2 and IL-6 in this study.

PGE_2 is an essential factor in osteoclastogenesis. Osteoclast formation stimulated by 1,25-$(OH)_2$vitamin D_3 or PTH was reduced 60% to 70% in marrow cultures of mice lacking the COX-2 gene relative to cultures from wild-type mice with the intact COX-2 gene (77). Inhibition of osteoclast formation was reversed by the addition of exogenous PGE_2.

PGE_2 also appears to have a role in estrogen-deficient bone loss. PGE_2 accelerated bone resorption in estrogen deficiency. In ovariectomized (OVX) mice, ODF expression in bone marrow stromal cells (78) and pre-B cells (79) was enhanced by elevated production of PGE_2 in bone marrow induced by increased concentrations of pro-inflammatory cytokines such as IL-1 and TNF-α (80, 81). In another study, Kawaguchi *et al.* (82) showed that ovariectomy potentiated PGE_2 production in mouse calvarial osteoblastic cell cultures. This evidence indicates that estrogen deficiency not only enhanced the local production of pro-inflammatory cytokines, but also increased the susceptibility of bone tissue to their catabolic actions.

PGE_2 effects on bone cell formation and maturation are mediated through different PGE_2 receptors. Four subtypes of PGE_2 receptors have been identified in bone: EP_1, EP_2, EP_3 ($EP_{3\alpha}$, $EP_{3\beta}$, and $EP_{3\gamma}$), and EP_4. These receptors transmit the effect of PGE_2 through different signaling pathways: EP_1 activates the 1,3,5-inositol phosphate (IP_3) second messenger through calcium mobilization, EP_2 and EP_4 through the activation of adenylate cyclase, and EP_3 by inhibition (α- and β-subtype) or inhibition/activation of adenylate cyclase subtype γ (83–87).

The differentiation and proliferation of osteoblast and osteoclast cells is achieved in part by interactions between PGE_2 and its receptors in osteoblastic and marrow stromal cells. For example, PGE_2 stimulated osteoblast differentiation (bone nodule formation), but inhibited its proliferation through the EP_1-IP_3 pathway in cells from young rats while it inhibited differentiation, but stimulated proliferation through the EP_2/EP_4-cAMP pathway in cells regardless of the age of the donor animal (88). Miyaura *et al.* (89) showed that PGE_2 stimulated bone resorption by a cAMP-dependent mechanism via the EP_4 receptor, and a marked reduction in PGE_2 induced bone resorption was found in the calvarial and long bone cultures from EP_4 gene knockout mice. In another study, Sakuma (90) showed that PGE_2 enhanced osteoclast formation through its EP_4 subtype on osteoblasts. Osteoblast-mediated bone resorption induced by PGE_2 also appears to involve EP_2. PGE_2 showed a modest effect on the expression of ODF mRNA in osteoblastic and bone marrow stromal cell cultures obtained from EP_2 knockout mice (91). Suzawa *et al.* (92) also showed that PGE-stimulated bone resorption is partially mediated by EP_2 through the induction of ODF via a cAMP signaling pathway in mouse calvarial cultures. The current understanding of PGE_2 indicates effects on osteoclastogenesis that are mediated by its EP_4 receptor; however, the EP_2 subtype may have a role in this process.

Though EP_4 has a major role in osteoclastogenesis, there are still pathways that mediate the formation of osteoclasts independent of this receptor. Sakuma and coworkers (90) reported that both 1,25-$(OH)_2$vitamin D_3 and PTH induced osteoclast formation in bone marrow cell cultures in an EP_4 knockout mouse model.

Similar to the PGs, leukotrienes (LTs), the metabolites of the 5-lipoxygenase (5-LO) pathway, also participate in the local control of bone metabolism (93–95). In most cases, these compounds are believed to be local regulators of bone metabolism by stimulating bone resorption and inhibiting bone formation. Gallwitz *et al.* (94) reported that LTC_4, LTD_4, and 5-HETE stimulated isolated avian osteoclasts to resorb bone. Meghji *et al.* (95) found that LTB_4, LTC_4, LTD_4, 5-HETE, and 12-HETE stimulated bone resorption in calvariae of mice at picomolar concentrations, whereas PGE_2 produced this stimulatory effect at 10 nM. When LTB_4 was injected over the calvaria of mice, there was a significant increase in bone resorption, osteoclast numbers, and eroded surfaces (96). Treatment with BWA4C, a specific inhibitor of 5-LO, resulted in a dramatic decrease in the

number of TRAP-positive mononucleated preosteoclasts compared with the control and sham-treated groups along the antagonist mandibular buccal cortex 4 days after extraction of the rat upper molars unilaterally (97). On the other hand, LTs also affect osteoblast formation so as to have an impact on bone formation. Ren and Dziak (93) demonstrated that LTB_4 inhibited cell proliferation in cultured osteoblasts isolated from rat calvaria in a dose-dependent manner, but it may also interact with PG to regulate osteoblast activity. In addition, the bone-forming capacity of osteoblasts was impaired when fetal rat calvarial cells were cultured in the presence of 5-LO metabolites (98). Since dietary fatty acids can modulate lipoxygenase synthesis, the elevated LTB_4 production associated with inflammatory conditions may be attenuated with long-chain n-3 fatty acids to reduce the bone loss that occurs in these disorders.

Dietary Lipids Alter Local Factors Controlling Bone and Cartilage Metabolism

Lipids play an important role in skeletal biology and bone health. Acidic phospholipids facilitate cartilage mineralization in growth plate (99), and PGs mediate messages from biomechanical forces (59) and aid in regulating anabolic factors, including IGFs (4), to support bone formation and resorption. Emerging evidence from human and animal research support the hypothesis that dietary lipids influence bone modeling and remodeling. Human studies indicate that the dietary intake of certain fatty acids could help to maintain bone mineral density in the elderly (100), and saturated fat intake was associated with greater bone density in children (101). Recent investigations with chicks and rats revealed that polyunsaturated fatty acids (PUFA) and conjugated linoleic acids (CLA) affect histomorphometric measurements of bone modeling (73, 74, 102).

Dietary fat may influence bone metabolism by altering PG biosynthesis (73, 74, 102). The PGs, locally produced from 20-carbon essential fatty acid precursors (20:4n-6 and 20:5n-3) in osteogenic cells, regulate both bone formation and bone resorption (59). In support of the relationships between dietary PUFA, PGs, and bone metabolism, Watkins *et al.* (73, 102) reported that dietary lipids modulated *ex vivo* bone PGE_2 production and the concentration of IGF-I in bone tissues, and led to altered bone formation rates in growing chicks and rats (102). In these experiments, animals given long-chain n-3 fatty acids tended to show an increased rate of bone formation, suggesting a stimulatory effect on osteoblastic activity. The favorable effect of n-3 fatty acids on bone modeling in growing animals is supported by the observation of reduced bone mineral loss in ovariectomized rats supplemented with eicosapentaenoic acid (EPA 20:5n-3) (103). The bone-sparing effect of 20:5n-3 may be associated with diminished bone resorption or increased bone formation.

The PGE_2 produced by osteoblasts stimulates IGF-I synthesis and affects its action to support an anabolic response in bone (51). Studies with dairy fats revealed that butter fat blended with corn oil reduced *ex vivo* bone PGE_2 production, elevated bone IGF-I concentration, and increased bone formation rates in chicks nearly 60% compared with those given diets higher in n-6 fatty acids (74). The responses observed in bone tissue suggest that moderating the action of n-6 fatty acids (linoleic acid) with long-chain n-3 fatty acids or CLA can benefit bone modeling.

The establishment of the significant role of PGs, especially PGE_2, in bone biology and physiology and the link between PGE_2 and dietary n-6 and n-3 fatty acids inspired our investigations on the potential interactions between dietary lipids and bone metabolism. Furthermore, it is believed that IGF-I is regulated by PGE_2 to modulate bone formation and bone resorption (4, 59). Recent studies in our laboratory with animals (chicks and rats) and cell cultures (primary avian chondrocytes and MC3T3-E1 osteoblastic-like cells) revealed that n-6 and n-3 PUFA modified fatty acid composition of tissues and cells, affected the capacity of bone to produce PGE_2 at the tissue level, and altered histomorphometric measurements of bone formation parameters (73, 74, 102, 104).

Several studies showed that dietary lipids transform the fatty acid composition of bone compartments, which would impact the local production of factors influencing bone modeling in experimental animals. Watkins *et al.* (73) reported that an increased production of bone PGE_2 in tibia of chicks given a semi-purified diet containing soybean oil high in n-6 PUFA was associated with a lower rate of bone formation compared with that of chicks given a low dietary ratio of n-6/n-3 fatty acids. Furthermore, dietary n-3 PUFA was reported to lower the concentration of arachidonic acid (AA) in bone (73) and cartilage (105), and depress *ex vivo* PGE_2 production in bone organ culture. Chicks fed a blend of menhaden oil plus safflower oil in a semi-purified diet had a lower concentration of AA, but higher concentrations of 20:5n-3 and 22:6n-3 in cortical bone polar lipids compared with those given soybean oil.

Li *et al.* (102) recently reported that dietary n-6 and n-3 PUFA treatments influenced not only PGE_2, but also IGF-I concentration and bone histomorphometric measurements in growing rats. *Ex vivo* PGE_2 production was higher in rats given a diet high in n-6 PUFA (n-6/n-3 = 7.3) and serum IGF-I concentration was suppressed compared with those in the high n-3 PUFA treatment (n-6/n-3 = 1.8). In addition, rats fed diets high in n-6 PUFA had a lower ash weight per millimeter bone length in humeri compared with those fed n-3 PUFA. These findings suggest that excessive consumption of n-6 PUFA could have a negative effect on bone metabolism by increasing bone resorptive activity through elevated endogenous production of PGE_2. The observations in rats are consistent with the results in the chicken.

In another rat feeding study, *ex vivo* PGE_2 production in bone organ culture was significantly reduced in rats given diets with a lower dietary ratio of n-6/n-3 fatty acids (n-6/n-3 = 1.2–2.6) compared with those on diets with a higher dietary ratio (n-6/n-3 = 10–24) (104). Regression analysis

revealed a significant positive correlation between *ex vivo* production of PGE_2 and the ratio of AA/EPA in bone, but a significant negative correlation between bone formation rate and either the ratio of AA/EPA or PGE_2 in bone. Moreover, the activity of serum bone-specific alkaline phosphatase was greater in rats given a diet high in n-3 fatty acids, which further supports the positive action of these fatty acid on bone formation. These results demonstrated that the dietary ratio of n-6/n-3 fatty acids modulates bone PGE_2 production and the activity of serum bone-specific alkaline phosphatase in growing rats.

Enrichment of avian epiphyseal chondrocytes with linoleic acid (LA), AA, and EPA affected collagen synthesis in a dose-dependent fashion (73, 106). The LA and AA treatments reduced collagen synthesis, but EPA appeared to stimulate synthesis. Production of PGE_2 was reduced by EPA treatment, while LA and AA increased PGE_2 relative to the "no fatty acid" enrichment. In our laboratory, experiments on the MC3T3-E1 osteoblast-like cell line revealed that PGE_2 biosynthesis was decreased in 20-day, postconfluent cultures upon treatment with EPA when compared with treatment with AA plus IL-1α (107). The EPA-treated cells tended to have increased levels of alkaline phosphatase activity and osteocalcin level when compared with cells supplemented with AA plus IL-1α. Hence, enrichment of osteoblast-like cells with n-6 PUFA resulted in greater capacity to synthesize PGE_2, which appears to decrease cell activity.

Investigations with PUFA and epiphyseal (growth) cartilage and chondrocytes also indicate that dietary lipids affect cartilage metabolism in influencing bone modeling. Experiments on growth cartilage demonstrated that this tissue selectively accumulates dietary fatty acids (105, 108). These results suggest that chondrocytes are either sensitive to excess n-6 fatty acids or to an overproduction of PGE_2 (106). Growth cartilage in children and young animals contains small amounts of n-6 fatty acids, but a relatively high concentration of 20:3n-9 (Mead acid) (109). The concentration of Mead acid is not reduced in growth cartilage of growing animals given diets adequate or enriched in linoleic acid (18:2n-6) (105). Furthermore, supplementation of growth cartilage chondrocytes with linoleic or arachidonic acids depressed collagen synthesis (106), but these cells showed greater collagen synthesis when enriched with long-chain n-3 fatty acids or CLA (110, 111).

Reported findings on modulation of PGE_2 biosynthesis in bone tend to corroborate our results with PUFA treatments. Several studies have shown an increase in osteoblastic bone formation markers when PGE_2 production is decreased or inhibited (62, 112–115). Alam *et al.* (116) reported in rat alveolar bone that dietary PUFA alters fatty acid composition and PGE_2 production. Moreover, rats given diets supplemented with the n-3 fatty acid DHA demonstrated increased bone marrow cellularity (117). The n-3 fatty acids significantly slowed orthodontic tooth movement, which is effected by PGE_2-induced bone resorption in rats compared with those given diets high in n-6 fatty acids (118). Fish oil significantly lowered serum IL-6, TNF-α, and PGE_2 compared with corn oil in the MRL/lpr autoimmune prone mice (a model for rheumatoid arthritis) (119). Vitamin E further potentiated the suppressive effect of n-3 fatty acids in this study. EPA also suppressed the expression of TNF mRNA in LPS-stimulated RAW 64.7 cells (a mouse macrophage cell line), presumably by suppressing PGE_2 production (120). These data support a positive role of n-3 fatty acids on the modulation of PGE_2 action in maintaining optimal bone health.

Dietary PUFA and Human Bone Health

Osteoporosis. Osteoporosis occurs in both women and men. Postmenopausal women have the greatest risk of developing this disease. Osteoporosis is a condition of decreased bone mass that is prevalent in postmenopausal women and places them at risk for fractures, representing a significant public health problem. Extensive research has been conducted to find a cure for the disease, however, optimizing bone development in the young and reducing bone resorption to maintain bone mass and restore skeletal integrity in the older are still the best means to control the disease. Although more effective treatments are becoming available today for osteoporosis, such as different formulations of hormone replacement therapy (HRT), bisphosphonates, and novel formulations of calcitonin, reducing the existing risk factors is still a preferred approach (121). The best deterrent for osteoporosis is for women to build strong bones early in life by consuming a well-balanced diet (vitamin D, calcium, n-6 and n-3 fatty acids, and phytochemicals) and to follow a routine exercise program pre- and postmenopause. Thus far, direct evidence of any beneficial effect of dietary n-3 fatty acids on human osteoporosis is still lacking. However, experiments using animal and cell culture models, and epidemiological data suggest promising applications of n-3 PUFA on this widespread public health problem.

The primary pathogenesis involved in postmenopausal osteoporosis is an uncoupling of bone formation and resorption, which means that the bone resorption rate exceeds that for bone formation. The effect of n-3 fatty acids on bone formation and/or bone resorption was examined in both normal and ovariectomized rats. Iwami-Morimoto *et al.* (122) studied alveolar bone resorption in 4-week-old rats given diets supplemented with 10% of either fish oil or corn oil for 6 weeks. Dietary fish oil reduced osteoclastic activity (OC number was only 60% of control) and subsequent alveolar bone resorption (80% of control). In ovariectomized female Sprague-Dawley rats, normal or low calcium diets with or without added EPA (approximately 160 mg/day/kg) were administered for 35 days (103). Bone weight of femur and tibia decreased significantly in the low calcium group, but the decrease was inhibited in the EPA group given low calcium. Bones from rats given EPA with a low calcium diet were as strong as those from rats on a normal diet in the

bone rupture test and were stronger than those from rats given a low calcium diet (103). These results suggest that an EPA-enriched diet prevented the loss of bone weight and strength caused by estrogen deficiency as would occur during postmenopause in women. In another study (123), sham-operated or ovariectomized 11-week-old female Sprague-Dawley rats were given diets supplemented with fatty acids (linoleic acid as control, a diester of γ-linolenic acid or EPA) with or without subcutaneous estrogen implant. The rats on the diester diets (containing diesters of γ-linolenic acid or EPA) and diester plus estrogen implant showed increased calcium/femur and reduced urinary deoxypyridinoline and hydroxyprydinoline excretion compared with the sham-operated. Consequently, n-3 fatty acids worked synergistically with estrogen to exert a stimulatory effect on bone mineral deposition and an inhibitory effect on bone resorption.

These studies suggest that using n-3 fatty acid supplements, which are antagonistic to AA in the sense of eicosanoid action, could help maintain bone mineral content after menopause in women. Considering the results from these ovariectomized rat studies (inhibitory to bone resorption) and our findings on n-3 fatty acids in bone modeling (promoting bone formation), it is plausible that consuming diets rich in n-3 fatty acids will help to build and maintain a healthy skeleton in the human.

Inflammatory and Degenerative Bone/Joint Disease. Although osteoporosis is a major disease for postmenopausal women, rheumatoid arthritis (RA) and osteoarthritis (OA) affect millions of people worldwide. Rheumatoid arthritis afflicts peripheral joints in a symmetric distribution and osteoarthritis occurs mainly in weight-bearing joints. The bone and cartilage loss taking place in these disease processes is induced by eicosanoids, lymphokines, and free radicals, which also modulate immune response, influence cell proliferation, and stimulate collagenase and protease secretion (124).

Inflammatory cytokines (e.g. IL-1, a major player in OA and RA) are known to inhibit chondrocyte proliferation (125) and induce cartilage degradation, for which part of the response may be mediated by PGE_2 (126). Excess production of PGE_2 is linked to joint pathology (rheumatoid arthritis), known to exacerbate inflammatory responses, and results in a net loss of proteoglycan from articular cartilage (126). Elevated production of PGE_2 also has been shown to be associated with several osteolytic disorders in human, including bone loss associated with dental cysts (127), failing joint prostheses (128), and certain neoplasms (58, 129). The fact that selective COX-2 inhibitors gave satisfactory relief of symptoms in both osteoarthritis and rheumatoid patients (130, 131) suggests that eicosanoids participate in the inflammatory process of these severe bone/joint diseases. COX-2 and its product PGE_2 appear to be a common link between the two disease states and since it is possible to regulate the activity and expression of COX-2, this enzyme is a potential target for dietary intervention in optimizing bone formation and controlling bone disease.

Dietary intervention with n-3 fatty acids demonstrates consistent positive effects on inflammatory joint diseases. n-3 PUFA acts as a competitive inhibitor of eicosanoid biosynthesis in the treatment of RA. Several studies evaluating n-3 PUFA dietary supplements ranging from 3 to 6 g/day showed a modest, but rather consistent beneficial effect of these fatty acids in joint disease (132–141). At the same time, the syntheses of pro-inflammatory factors IL-1, IL-2, and TNF in cartilage tissue were suppressed by dietary supplementation with fish oil containing both EPA and DHA (142). Dietary fish oil supplementation given to rheumatoid arthritis patients resulted in a 19% to 20% reduction of neutrophil LTB_4 production from baseline and a decrease of 38.5% to 40.6% of macrophage IL-1 production (143). The use of flaxseed in domestic food preparation for 4 weeks also reduced the production of TNF-α and IL-1β by 30% in healthy volunteers (144). In addition, recent clinical trials indicate that some patients with RA are able to discontinue nonsteroidal anti-inflammatory drug NSAID use while receiving a source of n-3 fatty acids (145), suggesting that the mode of n-3 fatty acid action in RA patients could be related to eicosanoid biosynthesis. One explanation for this phenomenon is that the EPA metabolite PGE_3 is much less inflammatory compared with PGE_2 (146). Lowering PGE_2 in the diseased joint with diets rich in long-chain n-3 fatty acids could further benefit RA patients by reducing bone resorption since PGE_2 stimulates osteoclast activity, which results in secondary osteoporosis (145). Since PGE_2 activation of the IGF-I/IGFBP axis may play an important role in cartilage biology and collagen and proteoglycan synthesis (147), dietary fatty acids may also be important for supporting joint repair. Investigations are needed to describe the effects of nutraceutical fatty acids and the ratio of n-6/n-3 fatty acids on cartilage biology, joint disease, and ligament healing since dietary sources of these fatty acids exert potent effects on prostanoid biosynthesis in controlling cell activity in these processes.

New information on prostanoid formation and NSAID action resides in understanding the regulation and expression of cyclooxygenase. Two isoforms of this enzyme exist (COX-1 and COX-2). The COX- 1 is a constitutive enzyme responsible for generating PGs that act physiologically, while COX-2, an inducible enzyme, is stimulated by cytokines, growth factors, and tumor promoters. The COX-2 isoform is responsible for production of PGE_2 that is associated with inflammatory reactions (148) and cancer (149, 150).

Anti-inflammatory supplements, including nutraceutical n-3 fatty acids, are associated with decreased pathogenesis of rheumatoid arthritis (with subsequent secondary osteoporosis), reduced inflammatory diseases (141, 145, 151), and lowered cancer risk (152). Incorporation of n-3 fatty acids into articular cartilage chondrocytes membranes resulted in a dose-dependent reduction in the expression and

activity of proteoglycan degrading enzymes, the expression of inflammatory cytokines TNF-α and IL-1, and the expression of COX-2, but not COX-1 (153). A common link between these diseases resides in the regulation/expression of COX-2. For example, multiple lines of evidence indicate that upregulation of COX-2 contributes to tumorigenesis and inflammation (148, 154), providing tissue levels of prostanoid precursors that influence formation of the pro-inflammatory PGE_2. In addition, chronic aspirin (a COX inhibitor) users have reduced incidence of colorectal cancer (150). Both COX-1 and COX-2 inhibitors suppress experimental mouse skin carcinogenesis, and permanent over activation of arachidonic acid metabolism appears to be the driving force for tumor development (155). Moreover, metastasis of cancer to bone is a frequent outcome of breast (about two-thirds of patients with metastatic breast cancer have bone involvement) and prostate malignancies (156, 157). The metastasis is often associated with significant morbidity (severe bone pain and pathologic fractures) due to osteolysis, and the metastatic target bone is continually being remodeled under the influence of local and systemic factors (158).

Interactions Between PUFA and Other Dietary Factors: Phytoestrogen Isoflavonoids

Dietary factors such as calcium and vitamin D have long been linked to the prevention of osteoporosis, a disease associated with decreased bone content and increased bone fracture risk. However, despite the essential role of these two factors, populations with the highest calcium consumption also have higher osteoporosis-related incidence of limb fractures in the world (159, 160). It is logical to hypothesize, therefore, that other dietary factors present in foods have beneficial effects on bone health in those populations with relatively low rates of osteoporosis. Epidemiological studies indicate that populations consuming a diet high in long-chain n-3 fatty acids and soy products, a rich source of genistein, have a lower risk for cardiovascular disease and cancer, as well as osteoporosis (160–163).

Dietary phytoestrogens are plant-derived compounds that exert physiological effects similar to native estrogen. Genistein, a phytoestrogen rich in soy products, has been shown to be as active as estrogen in maintaining bone mass in ovariectomized rats (164). Moreover, genistein suppresses osteoclastic activity *in vitro* and *in vivo* (165), and the anabolic effect of genistein on bone compartments was inhibited by tamoxifen, an anti-estrogen agent, which further confirmed that the effect of genistein may be mediated through an estrogen-like action (166). Furthermore, isoflavones (e.g., genistein) possess antioxidant properties (167, 168) and they may contribute to better bone health by reducing the formation of free radicals and lipid peroxides that depress osteoblastic activity (110, 111). Therefore, one could speculate that phytoestrogens may serve as an anti-resorptive agent to decrease bone resorption, which would help maintain bone mass postmenopause. Although the research on soy and bone health is promising, investigators have yet to establish a clear hypothesis for its action and more recent studies suggest a beneficial effect on bone formation.

Genistein has a potent inhibitory effect on PTH-, PGE_2- and vitamin D-induced osteoclast-like cell formation in mouse marrow cultures (169). Genistein, acting also as a protein tyrosine kinase inhibitor, selectively reduced COX-2 expression without modification of COX-1 after induction by PMA (phorbol ester activator of PKC) and IL-1 (bone resorption inducer) in human endothelial cells (170). Other evidence also indicates that genistein could inhibit the expression of COX-2 in various cell systems. For example, genistein prevented IL-1 induced expression of COX-2 and production of PGE_2 in human islets (171), and inhibited PGE_2 synthesis in NRK cells (a fibroblastic clone) when induced by EGF (172). Genistein also inhibited COX-2 synthesis and activity in rats with endotoxic shock (173), completely blocked the production of PGE_2 in macrophages stimulated by LPS (174), and reduced COX activity in endothelial cells from newborn pig (175). These findings support the hypothesis that genistein has a general negative effect on PGE_2 production, which may downregulate osteoclastic activity or moderate prostanoid production to favor bone formation.

Summary

Significant changes have occurred over the past few decades in the fat composition of the food supply, in agricultural methods of food production, and in the eating habits of industrialized societies. These changes upset the dietary balance of n-6 to n-3 fatty acids consumed prior to the industrialization era. Based on food disappearance data (per capita food disappearance data from the USDA), the dietary ratio of n-6/n-3 fatty acids has decreased from 12.4:1 in 1985 to 10.6:1 in the U.S. in 1994 (176). However, the present dietary ratio of n-6/n-3 fatty acids may be far from the optimal recommended dietary intake of n-3 fatty acids to protect against chronic disease risk (177).

This review presented information that n-3 fatty acids improved bone metabolism in animals and reduced the symptoms of certain bone/joint diseases in the human. The positive effects of long-chain n-3 PUFA appear to be, in part, associated with downregulating PGE_2 formation in *ex vivo* rat bone organ culture and in osteoblast-like cell cultures. The modulation of prostanoid synthesis from arachidonic acid is linked to increased bone formation in growing rats and osteoblast cell activity in culture. The prospect of new n-3 fatty acid research that evaluates effects on biochemical and molecular factors involved in osteoblast differentiation, bone modeling/remodeling, and disease processes may reveal novel roles for these nutraceutical fatty acids in skeletal health.

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