# Vitamin A Metabolism, Action, and Role in Skeletal Homeostasis

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Vitamin A (retinol) is ingested as either retinyl esters or carotenoids and metabolized to active compounds such as 11-*cis*-retinal, which is important for vision, and all-*trans*-retinoic acid, which is the primary mediator of biological actions of vitamin A. All-*trans*-retinoic acid binds to retinoic acid receptors (RARs), which heterodimerize with retinoid X receptors. RAR-retinoid X receptor heterodimers function as transcription factors, binding RAR-responsive elements in promoters of different genes. Numerous cellular functions, including bone cell functions, are mediated by vitamin A; however, it has long been recognized that increased levels of vitamin A can have deleterious effects on bone, resulting in increased skeletal fragility. Bone mass is dependent on the balance between bone resorption and bone formation. A decrease in bone mass may be caused by either an excess of resorption or decreased bone formation. Early studies indicated that the primary skeletal effect of vitamin A was to increase bone resorption, but later studies have shown that vitamin A can not only stimulate the formation of bone-resorbing osteoclasts but also inhibit their formation. Effects of vitamin A on bone formation have not been studied in as great a detail and are not as well characterized as effects on bone resorption. Several epidemiological studies have shown an association between vitamin A, decreased bone mass, and osteoporotic fractures, but the data are not conclusive because other studies have found no associations, and some studies have suggested that vitamin A primarily promotes skeletal health. In this presentation, we have summarized how vitamin A is absorbed and metabolized and how it functions intracellularly. Vitamin A deficiency and excess are introduced, and detailed descriptions of clinical and preclinical studies of the effects of vitamin A on the skeleton are presented. **(*Endocrine Reviews* 34: 766–797, 2013)**



Abbreviations: *Akp1*, gene encoding alkaline phosphatase; AP-1, activator protein-1; APL, acute promyelocytic leukemia; ATRA, all-*trans*-retinoic acid; Bcl6, B-cell lymphoma 6; BMD, bone mineral density; BMP, bone morphogenetic protein; *Calcr*, gene encoding calcitonin receptor; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; CRE, cAMP response element; CREB, CRE binding; *Ctsk*, gene encoding cathepsin K; DAP12, DNAX-activating protein of 12 kDa; Dc-stamp, dendritic cell-specific transmembrane protein; DXA, dual-energy x-ray absorptiometry; FABP, fatty acid binding protein; FcR$\gamma$, Fc receptor common $\gamma$-subunit; FOP, fibrodysplasia ossificans progressiva; I$\kappa$B$\alpha$, inhibitory $\kappa$B $\alpha$; IKK$\beta$, inhibitor of NF-$\kappa$B kinase $\beta$; IRF-8, interferon regulatory factor-8; MafB, v-maf musculoaponeurotic fibrosarcoma oncogene homolog B; M-CSF, macrophage colony-stimulating factor; MITF, microphthalmia-associated transcription factor; NF-$\kappa$B, nuclear factor-$\kappa$B; Nfatc1, nuclear factor of activated T cells c1; 1,25$(OH)_2$ D3, 1,25-dihydroxyvitamin D; 25(OH) D, 25-hydroxyvitamin D; OPG, osteoprotegerin; OSCAR, osteoclast-associated receptor; PI3K, phosphoinositide 3-kinase; PLC$\gamma$, phospholipase C$\gamma$; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; 9-*cis* RA, 9-*cis* retinoic acid; RANK, receptor activator of NF-$\kappa$B; RANKL, RANK ligand; RAR, retinoic acid receptor; RARE, retinoic acid response element; RBP, retinol binding protein; RDA, recommended daily allowance; RE, retinol equivalent; ROR, retinoic-related orphan receptor; RORE, ROR responsive element; RXR, retinoid X receptor; TRAF, TNF-related associated factor; TRAP, tartrate-resistant acid phosphatase.

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## I. Introduction

Vitamin A is a vital nutrient known best for being required for good vision. Numerous over-the-counter preparations of vitamin A are available, and vitamin A is normally found in multivitamins. In the United States, it is not unusual for foods such as cereals to be heavily fortified with vitamin A. The pursuit of a healthy lifestyle often includes a diet where many foods contain vitamin A, as well as taking vitamin A supplements. Although the data are not conclusive, it has been suggested that increased intake of vitamin A may lead to osteoporosis and fracture in countries such as the United States, and in Scandinavia, where the intake of vitamin A in foods and from supplements is often high.

This review outlines how vitamin A is absorbed and handled by the body, both extracellularly and intracellularly; discusses general actions of vitamin A and mechanisms by which these actions are mediated; covers vitamin A deficiency and hypervitaminosis A; focuses on the clinical data for and against increased intake of vitamin A promoting skeletal fragility; and presents a detailed description, with historical perspective, of in vitro and in vivo experimental evidence for vitamin A effects on bone modeling and remodeling.

## II. Necessity of Vitamin A for Vision and Cellular Functions

The term "vitamin A" is used for any compound possessing the biological activity of retinol (retinyl esters, retinol, retinal, retinoic acid, and oxidated and conjugated forms of both retinol and retinal) (Figure 1). The term "retinoid" was introduced by Sporn et al (1) in 1976 and includes compounds consisting of 4 isoprenoid units joined in a head-to-tail manner. The term "retinoid" includes both naturally occurring forms of vitamin A and synthetic analogs of retinol.

Vitamin A has a number of important functions in the body. One is vision, because vitamin A is the precursor for the formation of 11-*cis*-retinal (2, 3). Rhodopsin, the light-sensitive pigment in rods of the eye, is formed when 11-*cis*-retinal combines with the protein opsin. Absorption of light energy causes rhodopsin to decompose by a series of photochemical reactions to all-*trans*-retinal and opsin. As this occurs, a visual signal is transmitted to the central nervous system. Night blindness is an early symptom of vitamin A deficiency (4). In night blindness, the small amount of light at night does not elicit an adequate response because the amounts of 11-*cis*-retinal and rhodopsin that can be formed are depressed. Another important function of vitamin A is regulation of growth and differentiation of cells. In the absence of vitamin A: 1) proper stem cell differentiation does not occur; 2) growth and development of embryos are altered; 3) epithelial cellular development is deficient, and the barrier to infection is decreased; 4) cells involved in innate and acquired immune function are decreased; 5) xerophthalmia develops because of abnormalities in corneal and conjunctiva development; and 6) normal bone growth and tooth development do not occur (5–11).

## III. Dietary Sources, Absorption, Hepatic Storage, and Transport of Vitamin A to Tissues

Vitamin A is obtained from the diet either as preformed vitamin A or as provitamin A carotenoids (12, 13). Preformed vitamin A is ingested as long-chained fatty acids of retinol (retinyl esters) in foods such as eggs, liver, butter, milk, and fortified cereals. Provitamin A carotenoids (eg, β-carotene, α-carotene, and β-cryptoxanthin) are found in vegetables such as carrots, spinach, collards, pumpkins, and squash. Ingestion of β-carotene is thought to account for over 75% of the provitamin A carotenoid intake in the United States. A retinol activity equivalent is equal to 1 μg retinol, 12 μg β-carotene, or 24 μg α-carotene or β-cryptoxanthin. Total vitamin A intake in the United States averages approximately 600 μg retinol activity equivalents per day. Of this, about 70–75% is thought to be due to the intake of preformed vitamin A.

Dietary retinyl esters are hydrolyzed by pancreatic and intestinal enzymes, and the free retinol is taken up by intestinal mucosal cells (ie, enterocytes; Figure 2) (14, 15). Retinol is insoluble in water, and in the enterocyte retinol is bound by

**Figure 1.**

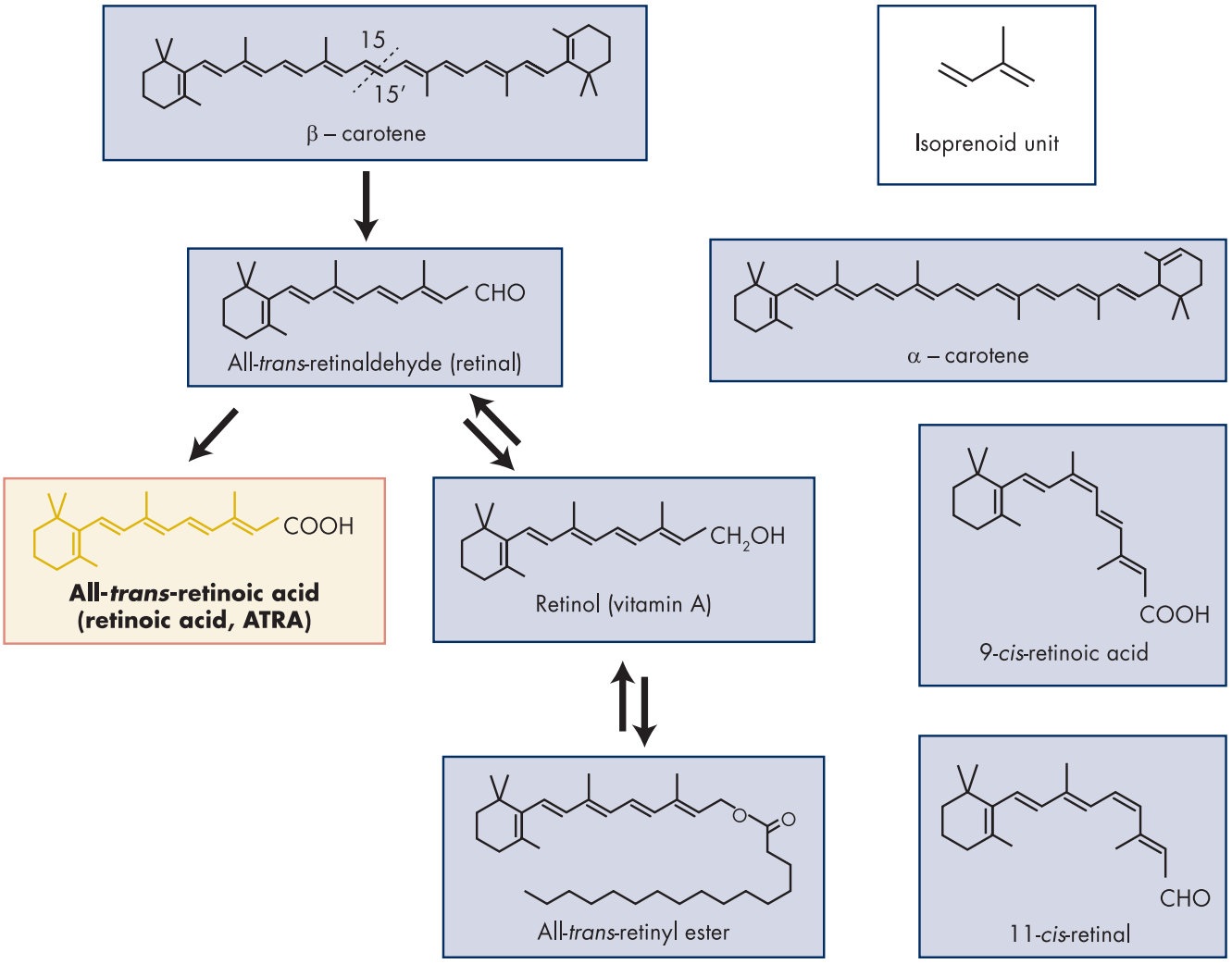


Figure 1. Metabolic interrelationships of retinyl ester, retinol, β-carotene, retinal, and retinoic acid, and the structures of the isoprenoid unit, α-carotene, 9-*cis* RA, and 11-*cis*-retinal. Most β-carotene is metabolized by central cleavage at the 15, 15′ double bond, yielding 2 molecules of retinal. Retinal, also called retinaldehyde, is interconvertible with retinol. Retinal also serves as an intermediate in the irreversible production of ATRA, which is considered the major biologically active derivative of vitamin A.

cellular retinol binding protein (CRBP) II. CRBPII is thought to bind most retinol in intestinal cells; it is 1 of 6 known retinoid binding proteins (13). About 50% of provitamin A carotenoids is absorbed intact in mucosa cells, and about 50% is oxidized to retinal and then reduced to retinol (16). Retinol derived from both retinyl esters and provitamin A carotenoids is esterified with long-chain fatty acids. The retinyl esters, together with intact carotenoids, are incorporated with other lipids (eg, cholesterol, cholesterol esters, and triglycerides) into chylomicrons, which are carried by the lymphatics (17). Some unesterified retinol is also believed to be absorbed directly by the portal system. The presence of fat in the diet greatly aids vitamin A absorption. Fat stimulates enzymes responsible for hydrolyzing dietary retinyl esters, increases micelle formation for solubilization of retinol and carotenoids in the intestinal lumen, and increases chylomicron formation (12).

In the bloodstream, chylomicron remnants containing retinyl esters are formed after hydrolysis of chylomicron triglyceride by lipoprotein lipase and addition of apolipoprotein E (13). Hepatocytes take up the remnants by receptor-mediated endocytosis, and the retinyl esters are hydrolyzed (18). If retinol is not needed by the body, it is reesterified and retained in liver stellate cells. The liver serves as the main storage depot of vitamin A (approximately 70% of total body stores). Smaller amounts of retinyl esters, as well as carotenoids, are also carried by chylomicrons and remnants to extrahepatic tissues for use and storage (13).

**Figure 2.**

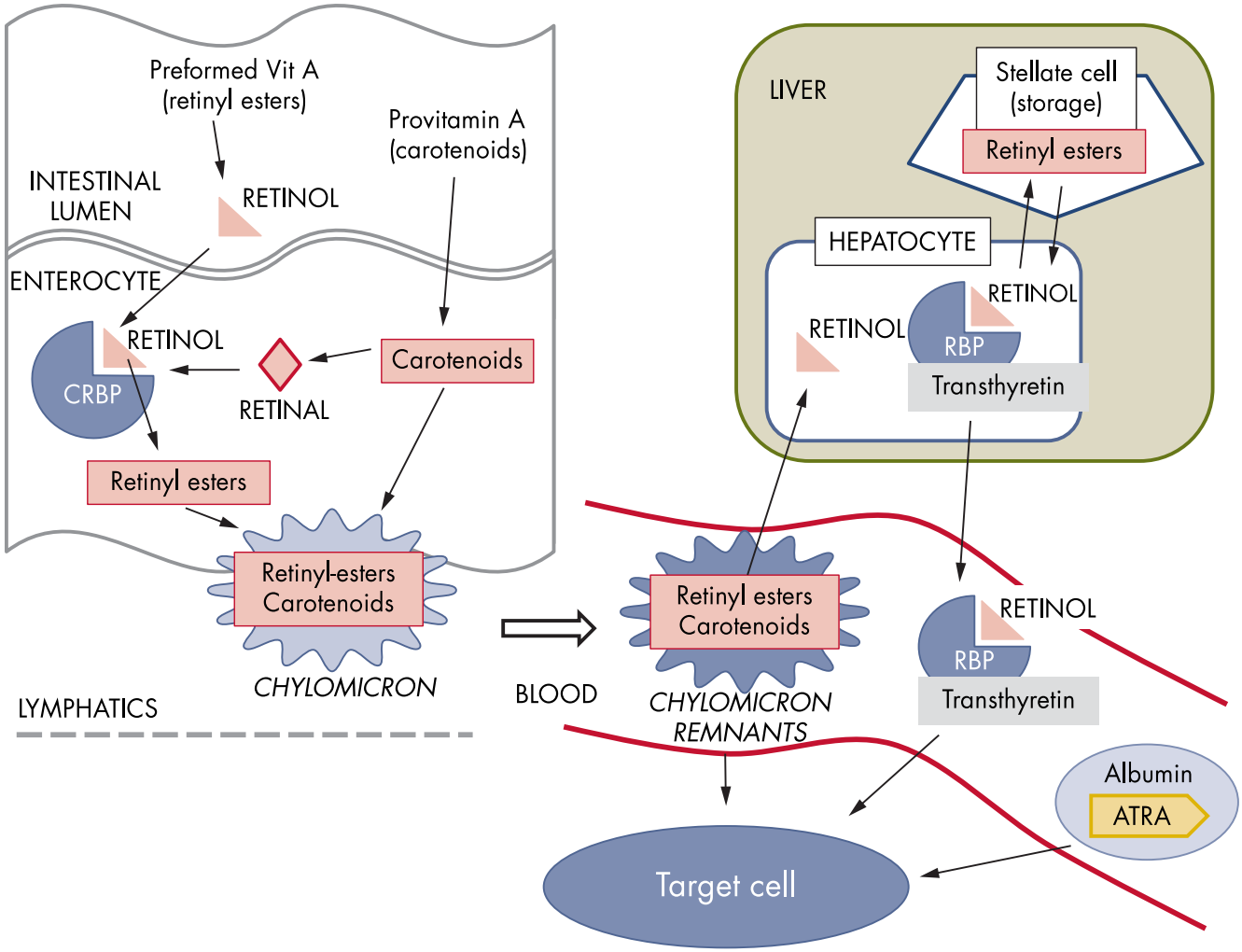


Figure 2. Dietary uptake and transport of vitamin A. Vitamin A is obtained from the diet as preformed vitamin A (retinyl esters) or as provitamin A (carotenoids, mainly β-carotene). Retinyl esters and carotenoids are incorporated into chylomicrons that are transported by the lymphatics. Chylomicron remnants can deliver retinoids directly to target cells, but the liver is the major organ for clearance of chylomicron remnants. Inside the hepatocytes, retinyl esters are hydrolyzed to retinol and bound to RBP. Retinol-RBP is combined with transthyretin and transported by the blood to target cells. If retinol is not needed, it is instead stored in liver stellate cells in the form of retinyl esters. Retinoids reach target cells mainly as retinol-RBP, but uptake of retinyl esters and carotenoids carried by chylomicrons and ATRA bound to albumin is also thought to occur.

After hydrolysis of retinyl esters in liver stellate cells, it is believed that retinol is transported back to hepatocytes and bound by a specific transport protein, retinol binding protein (RBP) (19). After combining with RBP, the retinol-RBP complex can enter the circulation, where it combines with transthyretin, a larger protein that is also synthesized in the liver (20). Binding of retinol-RBP to transthyretin prevents clearance of retinol-RBP by the kidney (21). In the fasting state, most retinoid in the circulation (>95%) is suggested to exist as retinol-RBP; however, in the postprandial state, chylomicron retinyl esters can account for a significant proportion of the total circulating retinoid (22).

## IV. Target Cell Uptake, Intracellular Metabolism, Cytoplasmic and Nuclear Receptors Mediating Vitamin A Effects

Peripheral cells take up retinoids mainly as all-*trans* retinol bound to RBPs in plasma. However, 25–33% of dietary retinoid that is absorbed in the intestine is delivered to tissues other than the liver by chylomicrons (13). It has been reported that bone is the second most important organ for clearance of chylomicron remnants and that vitamins can be delivered to osteoblasts in vivo via chylomicrons (23). Hydrolysis of chylomicron retinyl esters by lipoprotein lipase is thought to facilitate uptake of retinol

in tissues, whereas a transmembrane-spanning receptor encoded by the *Stra6* (stimulated by retinoic acid 6) gene is thought to be involved in the uptake of retinol bound to RBP (24) (Figure 3). In addition, the active metabolite all-*trans*-retinoic acid (ATRA) is present at low levels in serum bound to albumin and has been shown to contribute to tissue levels of ATRA (25).

Target cell metabolism of retinoids and the different signaling pathways of retinoids involve many different binding proteins and receptors (summarized in Figure 3). After cellular uptake, retinol is oxidized first to all-*trans* retinal by cytosolic alcohol dehydrogenases and bound to CRBP. Thereafter, all-*trans* retinal is oxidated by retinal dehydrogenases to the major biologically active metabolite, ATRA, which is subsequently bound by cellular retinoic acid binding proteins (CRABP).

Effects of retinoids are mediated primarily by 2 families of nuclear hormone receptors—retinoic acid receptors (RARs), and retinoid X receptors (RXRs) (26) (Figure 3). Each receptor family is made up of 3 isotypes ($\alpha$, $\beta$, and $\gamma$), produced by separate genes. Evaluation of different promoter usage and alternative splicing has shown that there are at least 2 different isoforms for each isotype (26). RARs form heterodimers with RXRs, and these heterodimers and RXR homodimers function as transcription factors, activating retinoic acid response elements

**Figure 3.**

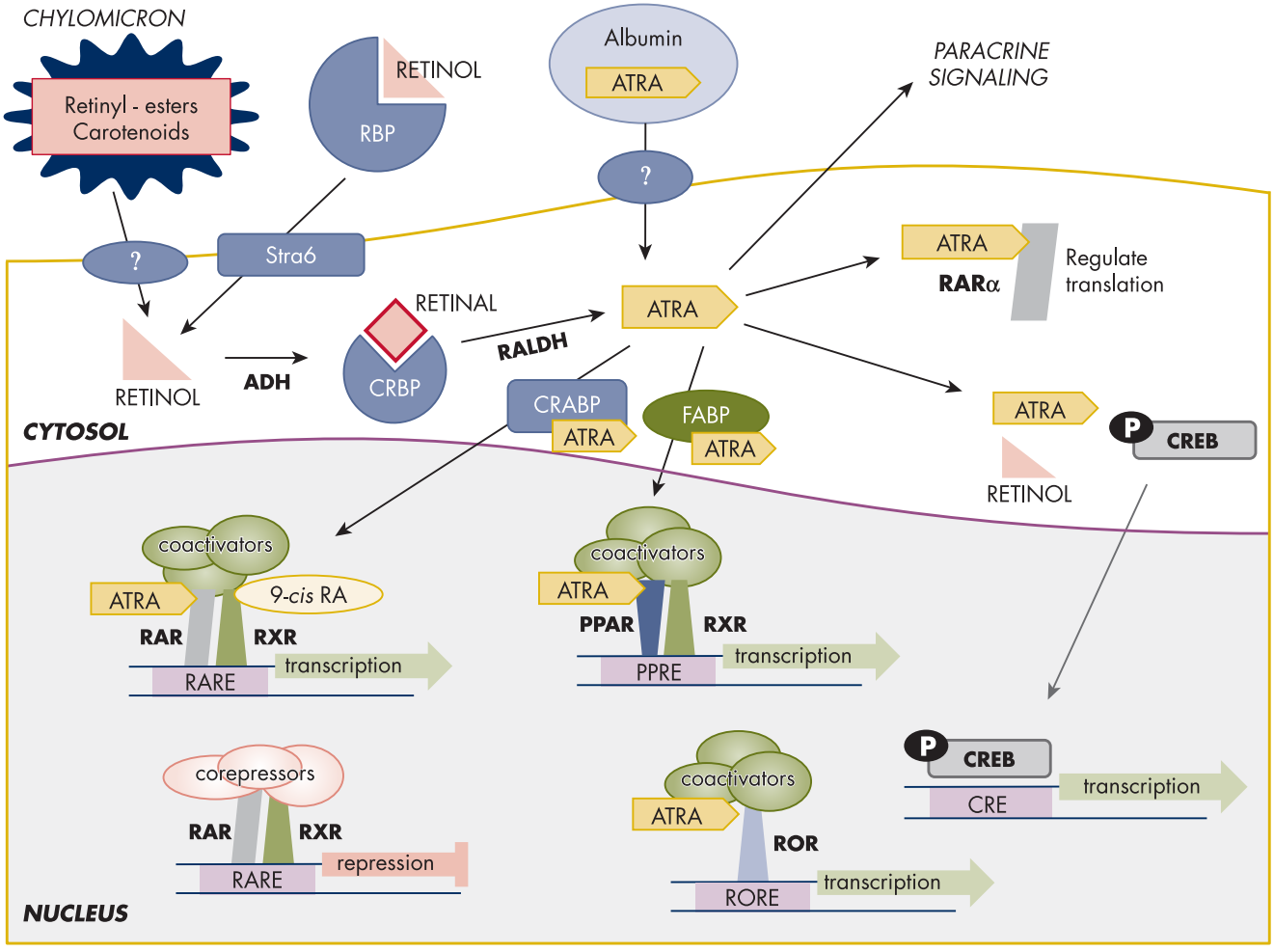


Figure 3. Target cell uptake and intracellular signaling. Retinoids reach target cells mainly as retinol bound to RBP. However, uptake of retinyl esters, carotenoids, and ATRA have also been described. Retinol is first oxidized to all-*trans*-retinal and bound to CRBP. Subsequently, all-*trans*-retinal is oxidized to ATRA, which is shuttled by CRABP to the nucleus where different RARs are activated. Alternatively, ATRA can be shuttled by FABP to bind PPARs in the nucleus. In addition to RARs and PPARs, ATRA can also bind to RORs to initiate transcription. In the absence of ligand, the RARs actively repress transcription. Nongenomic effects of ATRA include cytoplasmic regulation of translation and activation of signaling pathways causing phosphorylation of CREB and transcription of genes containing CRE. ADH, alcohol dehydrogenase; Stra6, stimulated by retinoic acid 6; RALDH, retinal dehydrogenase.

(RAREs) in the promoter regions of target genes (Figure 3). In vitro studies have shown that RARs can be activated by ATRA and the isomer 9-*cis* retinoic acid (9-*cis* RA), whereas RXRs are activated by 9-*cis* RA. Most retinol signaling in cells is thought to be mediated by ATRA binding RAR in RAR/RXR heterodimers (27). It is still not clear whether 9-*cis* RA is formed physiologically in bone cells and what role this isomer may play as a specific ligand for RXR (27); however, it was shown recently that 9-*cis* RA can be produced in vivo in the mouse pancreas (28), which suggests that the isomer may be physiologically relevant. Activated retinoid receptors function as transcription factors, activating specific RAREs for transcriptional regulation of target genes (26, 27). The RARE is a direct repeat of the hexamer motif PuG(G/T)TCA separated by 1 (DR1), 2 (DR2), or 5 (DR5) base pairs (26). Ligand binding of RARs is facilitated by CRABPII, which, upon shuttling ATRA to the nucleus, channels ATRA to RARs.

The RARs also have important regulatory functions in the absence of retinoids. When ligand is not present, the RAR/RXR actively represses transcription by recruiting corepressors such as nuclear receptor corepressor (NCoR), silencing mediator of RAR and thyroid hormone receptor (SMRT), mSin3A, and histone deacetylases (HDACs) (26, 29, 30) (Figure 3). RAR-mediated repression has been shown to be essential for chondroblast differentiation during skeletal development. ATRA levels are lower in the upper portion of the growth plate, which contains resting/proliferating chondrocytes, in comparison to the lower portion, which contains maturing/hypertrophying chondrocytes (31–33). The binding of ATRA to the RAR/RXR complex induces a conformational change in the ligand binding domain of the receptor, which causes replacement of corepressors by coactivators, such as members of the steroid receptor coactivator (SRC)/p160 family and p300/CREB-binding protein (CBP) (26, 34).

Peroxisome proliferator-activated receptors (PPARs), $\alpha$, $\beta/\delta$, and $\gamma$, represent another group of nuclear hormone receptors that forms heterodimers with RXR. PPAR/RXR heterodimers also function as transcription factors, activating specific response elements of target genes (35–37) (Figure 3). Recent studies have shown that ATRA can not only bind RARs but also serve as a ligand for PPAR $\beta/\delta$ (38). The fatty acid binding protein (FABP) 5 can shuttle ATRA to the nucleus, where it activates PPAR $\beta/\delta$ that bind to PPAR response elements (PPREs) (39, 40). The hypothesis that RA can have opposing effects depending on CRABPII/RAR or FABP5/PPAR $\beta/\delta$ binding has been proposed for keratinocytes and carcinomas, but so far it has not been tested in bone cells.

In addition to RARs, RXRs, and PPARs, retinoids have been observed to bind retinoid-related orphan receptors (RORs) $\beta$ and $\gamma$ (41). In contrast to RARs and PPARs, RORs do not form heterodimers with RXR. RORs regulate gene transcription by binding as monomers to specific ROR response elements (ROREs) in DNA (42). Recently, ROR$\beta$ was shown to suppress mineralization and decrease expression of osteocalcin and osterix mRNA in cultured murine osteoblasts (43).

The activation and repression of transcription through ligation of ATRA to different nuclear receptors and binding to response elements (RARE, PPRE, and RORE) in the promoters of genes represent genomic effects of the retinoids. Retinoids can also have rapid nongenomic/nonclassical actions. ATRA induces a rapid phosphorylation of cAMP response element binding protein (CREB), which translocates to the nucleus, binds, and activates genes containing cAMP response elements (CREs) in their promoters (44) (Figure 3). This effect is not limited to ATRA but can also be exerted by retinol, and it is mediated by cytosolic kinases not involving RARs (45). Another example of nongenomic effects of retinoids is mediated by RAR$\alpha$. In neuronal cells, RAR$\alpha$ has been shown to be actively transported from the nucleus to the cytoplasm, where it functions as an RNA-binding protein, inhibiting translation of other proteins (46–48).

Besides serving as an intermediate in retinoic acid formation, retinal has been shown to be present at biologically active concentrations in fat tissue, where it antagonizes PPAR activity, inhibits adipogenesis, and improves insulin sensitivity (49). These observations suggest that retinal may be an additional vitamin A derivative that plays an important role as a mediator of biological processes.

In contrast to retinol, ATRA is partially soluble in water (210 nM), can diffuse through water-soluble phases and hydrophobic membranes, and may have paracrine effects. These properties help make it an ideal morphogen, and ATRA has important functions during embryogenesis and development (50, 51).

## V. Vitamin A Deficiency and Excess

Vitamin A deficiency is still a problem in developing countries, and supplementation with vitamin A has had an enormous worldwide impact, improving vision and immune functions and saving countless lives at a minimal cost per patient (52). There has also been widespread use of retinoids for treatment of various skin conditions, such as acne (53), and for different cancers, including acute promyelocytic leukemia (APL), Kaposi's sarcoma, head

and neck squamous cell carcinoma, ovarian carcinoma, and neuroblastoma (54).

Assessing vitamin A status in individuals is difficult. The most common methods involve determining serum retinol and retinyl ester concentrations. Because vitamin A is stored in the liver and released as needed bound to RBP, measurement of the serum retinol level is not a sensitive method for determining vitamin A status, except when levels are very low or very high (55). Serum levels of retinol increase with age and under normal conditions in adults are in the range of 1–3 $\mu$mol/L. Serum concentrations of retinol $<1.05$ $\mu$mol/L indicate potentially inadequate vitamin A status, and concentrations $<0.70$ $\mu$mol/L indicate deficiency (56, 57). The serum level of retinol is not associated with hepatic vitamin A storage over a wide range of liver values, and alternative methods, including dose-response tests and isotope dilution assays, have been developed that are better indicators of liver vitamin A reserves (55, 58–60). Measurement of serum retinol levels is also not adequate for determining vitamin A status in individuals with clinical or subclinical toxicity (61). Serum retinyl esters have been suggested as an alternative marker for chronic hypervitaminosis A (62, 63). Serum retinyl ester levels of 0.2 $\mu$mol/L or exceeding 10% of total serum vitamin A (retinol plus retinyl esters) have been suggested to reflect excess retinol stores and potential toxicity. In the Third National Health and Nutrition Examination Survey (NHANES III), the prevalence of serum samples with retinyl ester concentrations $>10\%$ of total vitamin A was substantially higher than expected (64). Elevated retinyl esters were not correlated with serum markers of abnormal liver function, but the data nevertheless suggest that mild hypervitaminosis A may be more common than expected. Serum retinyl ester concentrations are higher in women than in men and higher in users than nonusers of supplements containing vitamin A (63, 64). Serum retinyl esters also increase with age, an effect likely reflecting increased intestinal uptake and decreased clearance of chylomicron remnants (63–66).

Excessive intake of preformed vitamin A or retinoid derivatives can cause hypervitaminosis A; however, hypervitaminosis A does not occur after increased intake of provitamin A carotenoids such as $\beta$-carotene. The conversion of $\beta$-carotene to retinol is feedback regulated and decreases when adequate vitamin A is present (67). Yellowing of the skin, carotenemia, can occur with high intake of carotenes, but this is reversible when intake is decreased (68).

Excessive intake of preformed vitamin A may have been a problem since ancient times (69). Bone changes consistent with chronic hypervitaminosis A have been observed in a partial *Homo erectus* skeleton found in Kenya. Sections of the tibial shaft showed pathology confined to the outermost cortex, with no evidence of abnormal remodeling of the underlying bone. Eating of animal livers, during a period of time when the dietary habit of *Homo erectus* was changing, was suggested to be responsible for the high intake of vitamin A.

Acute hypervitaminosis A sickness, characterized by vertigo, vomiting, diarrhea, headache, convulsions, and peeling of the skin, also occurs. An early explorer, Gerrit de Veer, is believed to be the first Westerner to describe acute hypervitaminosis A (70). He was a Dutch officer on William Barentsz' third voyage (in 1596) in search of the Northwest Passage. In his diary, he describes the sickness developing after the consumption of polar bear liver, which is extremely high in vitamin A. Elisha Kent Kane, an Arctic explorer in the 1850s, upon being told that polar bear liver was poisonous, thought it was a "vulgar prejudice," but he later reported, "The cub's liver was my supper last night, and to-day I have the symptoms of poison in full measure—vertigo, diarrhea, and their concomitants" (71). In another account, acute hypervitaminosis A developing after ingestion of Greenland Husky sled dog liver (also quite high in vitamin A) may have been responsible for the sickness and death that occurred in polar explorers of the Far Eastern Party, part of the 1911–1914 Australasian Antarctic Expedition (72).

The development of hypervitaminosis A is rare today. It is seen more often in children than in adults, often associated with retinoid treatment or candy-like supplements (73), but with the current interest in healthy lifestyle and the over-the-counter availability of vitamin A preparations, the potential exists for toxicity to become an ever-increasing problem. Consumption of water-miscible, emulsified, and solid forms of retinol is thought to pose the greatest threat (74).

Characteristic skeletal changes due to hypervitaminosis A in experimental animals are thinning of the cortex of long bones at the diaphysis and an increased frequency of fracture. Short-term studies in rodents treated with increased concentrations of retinoids (ATRA or Ro 13–6298) suggest that cortical bone thinning is due primarily to stimulation of subperiosteal resorption (75). In rats exhibiting hypervitaminosis A with thin, weakened long bones and increased periosteal osteoclasts, reduced endosteal/marrow blood flow and pathological endosteal mineralization have also been reported (76). Although most animal studies have been performed using higher dosages of vitamin A than humans would normally be exposed to, in vivo effects do not always require such high concentrations of vitamin A. Thinning of long bones and decreased biomechanical strength have been reported in

mature rats at lower "subclinical" hypervitaminosis A dosages (77). These data suggest that an increased incidence of osteoporosis and fractures could occur in humans with subclinically increased levels of vitamin A.

Skeletal effects noted in case studies of humans with significantly increased intake of retinoids include premature epiphyseal closure, skeletal and joint pain, increased cortical bone resorption, and periosteal bone formation associated with a characteristic hyperostosis in metatarsals, metacarpals, and long bones of the body (78–81). A microradiographic study of bone taken from a patient's rib during a period of high vitamin A intake showed bone resorption surfaces 6-fold above control values, accompanied by low normal bone formation (82). After normalization of high vitamin A intake, human radiographs have shown new subperiosteal bone formation in affected areas, such as the shafts of tibias and fibulas (83). Interestingly, numerous case reports of hypervitaminosis A have indicated the presence of hypercalcemia (84–87). Furthermore, it has been observed that the hypercalcemia can be decreased by glucocorticoid treatment (84, 87, 88). Recent studies have shown that glucocorticoids oppose the in vitro bone resorptive effect of ATRA by activating the monomeric glucocorticoid receptor (89). The hypercalcemia noted in hypervitaminosis A patients is thought to be due to a direct bone resorptive action of retinoids, suggesting that the mechanism by which glucocorticoids are decreasing hypercalcemia in patients exhibiting hypervitaminosis A may be mediated by the monomeric glucocorticoid receptor.

## VI. Human Studies Evaluating Whether Current Intake of Vitamin A Is Associated With Osteoporosis and Fracture

The current recommended daily allowance (RDA) of vitamin A is 900 $\mu$g/d in adult males and 700 $\mu$g/d in adult, nonpregnant, or nonlactating females. The tolerable upper level of vitamin A, the highest level likely to pose no ill effects, is 3000 $\mu$g/d in adult males and females (90). Clinical studies investigating the association between vitamin A and osteoporosis or fracture risk have suggested that vitamin A can be both harmful and beneficial to bone. The studies are mainly observational, and as stated above, it is difficult to determine vitamin A status in individuals. Results can also be influenced by the vitamin D status. The following sections and Table 1 summarize the clinical studies in relation to their reported effects on osteoporosis and fracture.

### A. Studies suggesting an association of increased vitamin A intake with osteoporosis and fracture

A number of studies have suggested that increased osteoporosis and fracture may occur with "subclinical" levels of vitamin A. Melhus et al (91) observed an increase in hip fracture for dietary intakes of retinol $>1500$ $\mu$g/d vs $\leq 500$ $\mu$g/d (odds ratio, 2.1) in a nested case-control study where 247 women with a first hip fracture were age-matched with 873 controls from a cohort of 66 651 Swedish women. In contrast to retinol, intake of $\beta$-carotene was not associated with an increased risk of hip fracture. In clinical studies, fracture risk is considered to be the best predictor of bone health, but measurement of bone mineral density (BMD) is also commonly employed for evaluating bone, and in a cross-sectional study of 175 randomly selected Swedish women 28–74 years of age, Melhus et al (91) showed that BMD was decreased 6–14% at skeletal sites (femoral neck, Wards triangle, trochanter region of proximal femur, lumbar spine, and total body) for retinol intake $>1500$ $\mu$g/d vs $\leq 500$ $\mu$g/d. Furthermore, a longitudinal, population-based, prospective study of 2322 men in Uppsala, Sweden, with 30 years of follow-up has suggested that the risk of fracture, including hip fracture, is higher among men with the highest levels of serum retinol (92). When the risk of fracture among subjects with an estimated dietary intake $>1500$ $\mu$g/d was compared to those with estimated intakes $<500$ $\mu$g/d, it was found that the risk of fracture was increased by a factor of approximately 2 among subjects in the highest category of vitamin A intake. No risk of fracture was noted with $\beta$-carotene intake.

An 18-year prospective analysis of vitamin A intake and hip fractures among 72 337 postmenopausal women in the Nurses' Health Study in the United States has also suggested a detrimental effect of increased vitamin A intake on bone health (93). Women in the highest quintile of vitamin A intake ($\geq 3000$ $\mu$g/d) had a significantly increased relative risk of 1.48 for hip fracture in comparison to women in the lowest quintile of intake ($\leq 1250$ $\mu$g/d). The increased fracture risk was attributed to increased retinol intake, with no significantly increased risk of fracture noted for $\beta$-carotene intake. Use of supplements alone was associated (although nonsignificantly) with a 40% increase in risk.

The Rancho Bernardo Study, a study of retinol intake and BMD in the elderly, has presented additional evidence in support of bone fragility (94). In this investigation, BMD of total hip, femoral neck, and spine at baseline and after 4 years was measured in 570 women and 288 men. The mean age at follow-up was 71 years. Both high and low retinol intakes were associated with decreased BMD at baseline and at 4-year follow-up. Maximum BMD was observed at retinol intakes of approximately 600–840 $\mu$g/d. Increased retinol

**Table 1.** Human Studies Evaluating the Risk of Fractures and BMD to Determine the Impact of Increased Vitamin A Intake on Bone Health

| Ref. | Subjects | Risk of Fracture | BMD |
|---|---|---|---|
| Studies suggesting an association between increased vitamin A intake and osteoporosis or fracture | | | |
| 91 | 247 Women with a first hip fracture age matched with 873 controls from a cohort of 66 651 Swedish women | Odds ratio of 2.1 for retinol intakes of >1500 μg/d vs ≤500 μg/d; no increased risk of hip fracture with β-carotene intake | |
| | 175 randomly selected Swedish women 28–74 y of age | | BMD decreased 14% at skeletal sites (femoral neck, Wards triangle, trochanter region of proximal femur, lumbar spine, and total body) for intake >1500 μg/d vs ≤500 μg/d |
| 92 | Longitudinal, population-based, prospective study of 2322 men in Uppsala, Sweden, with 30-y follow-up | Risk of fracture, including hip fracture, was increased by approximately a factor of 2 for dietary retinol intakes of >1500 μg/d vs <500 μg/d; no increased risk of fracture with β-carotene intake | |
| 93 | 18-y prospective analysis involving 72 337 postmenopausal women in the United States—the Nurse's Health Study | Relative risk of hip fracture for vitamin A intake ≥3000 μg/d vs ≤1250 μg/d was 1.48; no increased risk of fracture with β-carotene intake | |
| 94 | Prospective analysis involving 570 men and 288 women, with 4-y follow-up—the Rancho Bernardo study; mean age at follow-up, 71 y | | BMD measured in total hip, femoral neck, and spine; high and low retinol intakes were associated with decreased BMD at baseline and follow-up, with increased retinol intake becoming negatively associated with bone health not far beyond the RDA; intake reached predominately by supplement users |
| 95 | Fracture risk in 2799 women 50–74 y of age in the NHANES 1 follow-up study | Hip fracture was increased in both the lowest and highest vitamin A quintiles, with individuals in the highest quintile (≥73.3 μg/dL) having more than twice the risk of women in the middle quintile | |
| 96 | 3052 Norway women 50–70 y of age in a substudy of the population-based Nord-Trondelag Health Study | | Higher BMD of the distal forearm found in women with no childhood cod liver oil intake vs women taking cod liver oil in childhood |
| Studies suggesting only a weak relationship, at best, between increased vitamin A intake and osteoporosis or fracture | | | |
| 97 | Prospective analysis of 34 703 postmenopausal women; the Iowa Women's Health Study | Slightly increased risk of hip fractures among users of supplements, but no dose response or increased risk of all fractures with supplements, and no association between vitamin A or retinol intake from food and supplements, or food only, and the risk of hip or all fractures | |
| 98 | Longitudinal study of 891 women aged 45–55 at baseline and 50–59 after follow-up 5–7 y later | | Dietary vitamin A appeared to increase bone loss at the femoral neck, but there was no associated BMD loss when vitamin A from supplements (mostly cod liver oil) was added to dietary intake of vitamin A |
| 99 | Case-control study employing postmenopausal women with (n = 30) and without (n = 29) osteoporosis | | Total vitamin A intake did not differ between the 2 groups, but exceeded the RDA for intake by almost 2-fold in both groups; retinyl esters as a % of total serum vitamin A tended to be associated ($P = .07$) with osteoporosis |
| Studies showing no association of increased vitamin A intake to osteoporosis or fracture | | | |
| 100 | 11 068 women 50–79 y of age in the Women's Health Initiative Observational Study at 3 clinics in the United States | | No relationship of retinol or retinol + carotenoids from foods or foods and supplements to BMD |
| 101 | 470 men and 474 women 67–79 y of age in a prospective, population-based study in eastern United Kingdom | | No association was found between vitamin A or β-carotene intake and BMD loss; BMD loss (% per annum) was measured on 2 occasions (range, 2–5 y) |

(*Continued*)

**Table 1.** Continued

| Ref. | Subjects | Risk of Fracture | BMD |
|---|---|---|---|
| 102 | Cross-sectional study in 246 postmenopausal women in a rural Iowa community in the United States | No relationship between vitamin A supplement use or serum retinol to fracture risk | No relationship between vitamin A supplement use or serum retinol to radial bone mass |
| 104 | A population-based cohort of 2016 perimenopausal women in the Danish Osteoporosis Prevention study (DOPS) | No association of vitamin A intake to fracture risk | No association of vitamin A intake to lumbar spine and femoral neck BMD, or 5-y changes in BMD |
| 105 | 5709 males and females 20–90 y of age participating in NHANES III | | No associations between fasting retinyl esters and any measure of bone mineral status |
| 106 | Case-control Danish study with 124 655 patients with fractures and 373 962 age- and sex-matched controls | No increased risk of fracture associated with use of the retinoids isotretinoin and acitretin | |
| Studies suggesting a beneficial effect of vitamin A for bone health | | | |
| 107 | Nested case-control study (312 cases and 934 controls) from a cohort of 2606 women greater than 75 y old in the United Kingdom | Cod liver oil or multivitamin supplementation was associated with a significantly lower risk of any fracture | There was a tendency for increased serum retinol to predict benefit rather than harm in terms of BMD |
| 108 | Case-control study of Italian women 60 y of age and older, 75 with severe osteoporosis and 75 without osteoporosis | | Plasma retinol correlated positively with femoral neck BMD |
| 109 | Case-control study of Italian women 60 y of age and older, 45 with severe osteoporosis and 45 osteoporosis free | | Plasma retinol correlated positively with femoral neck BMD, but significance was not observed with carotenoids |
| 110 | 66 premenopausal women 28–39 y of age participating in a clinical trial in Tucson, AZ | | BMD measurements of total body, lumbar spine, and 3 femoral sites at baseline, 5, 12, and 18 months indicated that vitamin A and carotene from food slowed the annual rate of bone loss |
| Studies suggesting an association of increased vitamin A intake/low vitamin D with osteoporosis or fracture | | | |
| 115 | 75 747 postmenopausal women 50–79 y of age participating in the Women's Health Initiative Observational Study in the United States | Increases in total fracture risk noted with high vitamin A (≥7508 μg RE/d) and retinol intakes (≥1426 μg/d) with low vitamin D intake ≤11 μg/d; hazard ratios for high vitamin A and retinol intakes with low vitamin D were 1.19 and 1.15, respectively | |
| 116 | Cross-sectional study of 232 postmenopausal Spanish women with (n = 101) and without (n = 124) osteoporosis determined by quantitative ultrasound of the calcaneal bone. | | The prevalence of high serum retinol levels (≥2.8 μmol/L) was 36.4%; that of vitamin D deficiency, 70.1%; for individuals with vitamin D deficiency (n = 152), 60.4% (n = 92) had serum retinol levels higher than 2.8 μmol/L; in women with vitamin D deficiency, risk of osteoporosis in the highest retinol quintile was 5 times greater than risk in the lowest retinol quintile |
| 117 | Cross-sectional study of 232 postmenopausal Spanish women with (n = 78) and without (n = 154) osteoporosis determined by DXA of the lumbar spine and total hip | | The prevalence of high serum retinol levels (>80 μg/dL) was 36.4%; that of vitamin D deficiency, 70.1%; for individuals with vitamin D deficiency (n = 152), 60.4% (n = 92) had serum retinol levels higher than 80 μg/dL; in women with vitamin D deficiency, risk of osteoporosis was approximately 8 times higher in women with the highest retinol levels in comparison to women with the lowest retinol levels |

intake became negatively associated with bone health in both sexes not far beyond the RDA, intakes that were reached predominately by supplement users.

Data evaluating baseline serum vitamin A (retinol and retinyl esters) concentration quintiles and fracture risk in 2799 women 50 to 74 years of age in the first NHANES follow-up study have shown relationships similar to the Rancho Bernardo Study (95). Hip fracture was significantly elevated in both the lowest (≤46.1 μg/dL) and highest (≥73.3 μg/dL) vitamin A quintiles, with individuals in the highest quintile having more than twice the risk of a hip fracture than those in the middle quintile. The highest vitamin A quintile concentration (≥73.3 μg/dL) evaluated by the authors was similar to the highest vitamin A quintile (≥75.62 μg/dL) reported in the study by Michaelsson et al (92).

Supplementation with vitamin A during growth has also been associated with decreased bone mass in peri- and premenopausal women. In a substudy of the population-based Nord-Trøndelag Health Study, 3052

Norway women 50–70 years of age had BMD of the forearm measured. Women reporting no childhood cod liver oil intake had statistically higher BMD of the distal forearm than women reporting childhood intake of cod liver oil (96). Furthermore, there were indications of a negative dose-response effect of childhood cod liver oil intake on bone.

### B. Studies suggesting only a weak relationship, at best, between increased vitamin A intake and osteoporosis or fracture

A cohort of 34 703 postmenopausal women from the Iowa Women's Health Study was followed prospectively for 9.5 years to determine whether high levels of vitamin A and retinol intake through food and supplement use were associated with an increased risk of hip fracture or all fractures. Although there was a slightly increased risk of hip fracture among users of supplements, there was no apparent dose response and no increased risk of all fractures among supplement users. Furthermore, the authors found no association between vitamin A or retinol intake from food and supplements, or food only, and the risk of hip or all fractures (97). Food and supplemental vitamin A intake were also evaluated in a longitudinal study conducted on 891 women aged 45–55 at baseline and 50–59 after follow-up 5–7 years later (98). BMD of the lumbar spine and femoral neck showed that although dietary vitamin A (or retinol) appeared to worsen bone loss at the femoral neck, there was no relationship of BMD loss to either retinol or vitamin A intake when the vitamin A from supplements (mostly cod liver oil) was added. Cod liver oil also contains vitamin D, and the authors suggested that it was possible that the vitamin D contained in cod liver oil or perhaps polyunsaturated fatty acids in the cod liver oil may have been the reason there was no relationship of total dietary vitamin A to BMD.

Vitamin A intake, together with serum vitamin A (retinol plus retinyl esters), has also been determined in a case-control study employing postmenopausal women 48–83 years of age with (n = 30) and without (n = 29) osteoporosis. Although total vitamin A intake did not differ between the 2 groups, intake exceeded the 700 $\mu$g/d RDA for women in both the osteoporosis and control groups by almost 2-fold. Although there was no association between osteoporosis and retinol or total serum vitamin A (retinol plus retinyl esters), retinyl esters as a percentage of total vitamin A tended to be associated ($P = .070$) with osteoporosis (99).

### C. Studies showing no association of increased vitamin A intake to osteoporosis or fracture

A cross-sectional study measuring BMD of the total body, spine, and total hip (with subregions) on 11 068 women 50–79 years of age enrolled in the Women's Health Initiative Observational Study and Clinical Trial at 3 clinics in the United States (Pittsburgh, Pennsylvania; Birmingham, Alabama; and Tucson, Arizona) found no relationship of retinol or retinol plus provitamin A carotenoids from foods or foods and supplements to BMD (100). In another study with 470 white men and 474 white women 67–79 years of age recruited from a prospective population-based diet and cancer study (EPIC-Norfolk) in eastern United Kingdom, no association was found between vitamin A or $\beta$-carotene intake and BMD loss (101). BMD loss (percentage per annum) was measured on 2 occasions (range, 2–5 y). Furthermore, no statistically significant relationship between vitamin A supplement use or serum retinol to radial bone mass or fracture was reported in a study of 246 postmenopausal women 48 to 80 years of age in a rural Iowa community in the United States (102). More than 36% used a vitamin A supplement, and 8% consumed an amount in excess of 2000 retinol equivalents (REs)/d. A prospective, randomized, single-blind study conducted on 80 men 18–58 years old (40 receiving 7576 $\mu$g/d retinol palmitate and 40 receiving placebo for 2, 4, and 6 wk) has also shown no alterations of serum markers of bone turnover. Serum levels of osteocalcin were measured at 0 and 6 weeks, and serum bone-specific alkaline phosphatase and N-telopeptide of type 1 collagen were measured at 0, 2, 4, and 6 weeks (103). Moreover, in a population-based cohort of 2016 perimenopausal women in the Danish Osteoporosis Prevention Study, no association of vitamin A intake was found with cross-sectional BMD at the lumbar spine and femoral neck, 5-year changes in BMD, or fracture risk (104).

High serum retinyl levels have been suggested to be a marker for excessive vitamin A intake. Fasting serum retinyl esters and BMD were evaluated in the NHANES III, 1988–1994, a large, nationally representative sample of the U.S. population (105). BMD for the femoral neck, trochanter, intertrochanter, and total hip was measured in 5709 participants aged 20 to 90+ years. Although substantial prevalence of high retinyl esters and low BMD was noted, there were no significant associations between fasting retinyl esters and any measure of bone mineral status.

Risk of fracture with commonly employed vitamin A analogs has also been evaluated, and in a recent, large-scale, case-control Danish study, no increased risk of frac-

ture after use of isotretinoin and acitretin was observed (106).

### D. Studies suggesting a beneficial effect of vitamin A for bone health

Levels of serum retinyl ester (retinyl palmitate), together with serum retinol and β-carotene, were evaluated to determine whether they were predictors of osteoporotic fractures, including hip fracture, in a nested case-control study (312 cases and 934 controls) from a cohort of 2606 women more than 75 years of age in the United Kingdom (107). Although no evidence to support skeletal harm associated with increased serum indices of retinol exposure or modest retinol supplementation was found, there was a tendency for increased serum retinol to predict benefit rather than harm in terms of BMD. Furthermore, multivitamin or cod liver oil supplementation was associated with a significantly lower risk of any fracture. Other studies have also suggested that vitamin A was associated with enhanced skeletal health. In a case-control study of Italian women 60 years of age and older (75 with severe osteoporosis and 75 without osteoporosis), plasma retinol was found to be positively correlated with femoral neck BMD (108). In another study, plasma retinol, β-carotene, α-carotene, and β-cryptoxanthin were measured in a case-control study of Italian women 60 years of age and older (109). In this study group, 45 exhibited severe osteoporosis, and 45 were osteoporosis free. Plasma retinol was found to correlate positively with femoral neck BMD, but significance was not observed with the carotenoids. A prospective study measuring BMD of the total body, lumbar spine, and 3 femoral sites at baseline and 5, 12, and 18 months later in 66 premenopausal Caucasian women participating in a clinical trial in Tucson, Arizona, has also reported that vitamin A and carotene from food help slow the annual rate of total body bone loss (110). Similar findings have also been observed in a longitudinal study (111). Bone mineral content of the radius, ulna, and humerus was measured every 6 months for 3 years in 17 premenopausal women at the University of Wisconsin. A slowing of the rate of humerus bone loss was attributed to vitamin A intake.

### E. Studies suggesting an association of increased vitamin A intake/low vitamin D with osteoporosis or fracture

The RDA for vitamin D was increased in 2010 to 600 IU/d (15 μg/d) for individuals 1–70 years of age and 800 IU/d (20 μg/d) for older individuals (112). The serum concentration of 25-hydroxyvitamin D [25(OH) D] is the optimal clinical indicator of vitamin D status. Vitamin D deficiency is currently defined as 25(OH) D < 50 nmol/L (113).

A recent study employing pooled data from 11 double-blind, randomized, controlled trials of oral vitamin D supplementation offers a degree of validation to these recommendations. In 31 022 individuals 65 years of age or older (mean age, 76; 91% female) exhibiting 1111 hip fractures and 3770 nonvertebral fractures, median vitamin D intake of 800 IU/d (range, 792–2000) reduced the risk of hip fracture 30% and the risk of nonvertebral fracture 14% (114). Furthermore, analysis of serum 25(OH) D in a subgroup of the study (4383 individuals) was suggestive of serum 25(OH) D 60 nmol/L or greater decreasing fracture risk (114).

Some studies have suggested that bone fragility might be associated with increased vitamin A intake coupled to decreased vitamin D concentrations. In a longitudinal study evaluating the risk of hip and total fractures in 75 747 ethnically diverse postmenopausal women 50–79 years of age participating in the Women's Health Initiative Observational Study in the United States, no association between vitamin A or retinol intake and the risk of hip fractures or total fractures was observed; however, a modest increase in total fracture risk with high vitamin A (≥7508 μg RE/d) and retinol (≥1426 μg/d) intake was observed with low vitamin D intakes ≤11 μg/d (115). Hazard ratios for high vitamin A and retinol intakes with low vitamin D were 1.19 and 1.15, respectively. Furthermore, an association between low vitamin D and high serum retinol levels leading to an increased risk of osteoporosis has been reported recently in a cross-sectional study of 232 postmenopausal Spanish women (116). Osteoporosis was determined by quantitative ultrasound measurements of the calcaneal bone. Of the 232 women evaluated, 124 were designated as nonosteoporotic and 101 as osteoporotic. The prevalence of high serum retinol levels (≥2.8 μmol/L) was 36.4%. The prevalence of vitamin D deficiency [25(OH) D < 50 nmol/L] was 70.1%. For individuals with 25(OH) D < 50 nmol/L (n = 152), 60.4% (n = 92) had serum retinol levels higher than 2.8 μmol/L. A 3-fold increased risk of osteoporosis was observed in the highest retinol quintile. In women with vitamin D deficiency, risk in the highest retinol quintile increased to 5 times the risk in the lowest retinol quintile. In a second cross-sectional study of the Spanish women, 78 were found to be osteoporotic and 154 were nonosteoporotic, based on dual-energy x-ray absorptiometry (DXA) measurements (T-scores ≤ −2.5) of the lumbar spine and total hip. In this study, the risk of osteoporosis for women with vitamin D deficiency with the highest retinol levels

was suggested to be approximately 8 times greater than women with the lowest retinol levels (117).

Estimates of vitamin D status indicate widespread insufficiency. In Europe, mean vitamin D intake in Scandinavia has been reported to be 200–400 IU/d (118). This is significantly less than the currently recommended 600 IU/d, but it is believed to be more than twice the intake in other countries. A north-south gradient for 25(OH) D levels is thought to exist, with higher levels in Scandinavia and lower levels in countries like Italy and Spain (118). Higher 25(OH) D levels are found in Canada, where 35% of the population is thought to have levels >75 nmol/L (119). In the United States, it is estimated that approximately 60% of adults have serum levels >50 nmol/L, and approximately 30% have serum levels >75 nmol/L (120).

There have been numerous reports of vitamins D and A opposing one another. Increased vitamin D has been reported to protect the dog (121), chick (122), and human (74) against vitamin A toxicity, whereas excess vitamin A has been shown to reduce the effects of hypervitaminosis D in rats (123) and young broiler chickens (124). In humans, increased vitamin A intake is suggested to diminish the ability of vitamin D to increase calcium absorption (125). Although the reasons for the opposing effects of the active metabolites of vitamin A (ATRA) and vitamin D (1,25-dihydroxyvitamin D [1,25$(OH)_2$ D3]) are not known, their nuclear receptors, RAR and vitamin D receptor, respectively, employ the same heterodimerization partner, RXR, for binding to response elements of target genes (126, 127). Therefore, it is possible that high concentrations of either ATRA or 1,25$(OH)_2$ D3 might dampen heterodimerized receptor binding to responsive elements of DNA and subsequent actions of the other compound.

Levels of vitamin D have not been measured in most investigations evaluating vitamin A intake. In addition to the Women's Health Initiative Observational Study (115), the Nurses' Health Study (93) and the Iowa Women's Health Study (97) were 2 additional large prospective studies where vitamin D was measured. In the Nurses' Health Study, which suggested that increased vitamin A intake increased fracture risk, vitamin D intake averaged approximately 8 $\mu$g/d, which is lower than the 11 $\mu$g/d intake found to be associated with an increased risk of fracture in the Women's Health Initiative Observational Study. In contrast, in the Iowa Women's Health Study, which showed little, if any, relationship between vitamin A and fracture or all fractures, mean vitamin D intakes of supplement users and individuals in the fifth quintile of total vitamin A intake were 671 and 632 IU/d, respectively.

The clinical studies evaluating the skeletal risk associated with increased vitamin A intake have been based primarily on measurements of either fracture risk or BMD. Table 1 is a summary of these studies based on increases, decreases, or no association of fracture risk or BMD to increased vitamin A intake or increased vitamin A intake/low vitamin D. Present data suggest that increased vitamin A intake/low vitamin D favors a decrease in BMD and an increase in fracture risk; however, the effect of increased intake of vitamin A alone is less clear, with increases, decreases, and no associations to fracture risk and BMD reported.

## VII. Bone Remodeling and Modeling at the Cellular Level

In adult life, 2 processes are responsible for changes in the skeleton: remodeling and modeling (128). Remodeling is the process by which new bone replaces old bone. It goes on continuously in both cortical and trabecular bone. Under physiological conditions, the shape and mass of bone are not affected by remodeling. During remodeling, bone cells called osteoblasts form new bone at specific sites after old bone is resorbed by large, multinucleated cells termed osteoclasts. This is often referred to as coupling, and the sites where it occurs are called bone multicellular units. In contrast to remodeling, modeling of bone is the process by which new bone is formed without prior resorption, or where resorption occurs without subsequent bone formation. Both the shape and mass of bone can be changed by modeling.

### A. Bone resorption

Remodeling is initiated by formation of osteoclasts, mainly on endosteal surfaces of trabecular and cortical bone, or within the Haversian canals in cortical bone. It also occurs at periosteal surfaces of cortical bone, although less frequently. Fully differentiated osteoclasts are large, multinucleated cells that can be identified in histological sections by their expression of the enzymes tartrate-resistant acid phosphatase (TRAP) and cathepsin K. For resorption to occur, osteoclasts must first seal off an area of bone. Osteoclasts are believed to bind to bone surfaces where outer, nonmineralized osteoid has been removed (129). The breakdown (resorption) of bone is initiated by dissolution of bone mineral crystals by a lowered pH (approximately 4.5). Protons are pumped into the "resorption lacunae beneath the ruffled border" by a proton pump expressed in the ruffled border of osteoclasts. In

parallel with the mineral phase of bone being dissolved, osteoclasts release proteolytic enzymes that degrade type I collagen fibers and other noncollagen proteins in the bone matrix (130).

### B. Osteoclast proliferation, differentiation, and fusion

Multinucleated osteoclasts are formed by proliferation, differentiation, and fusion of mononuclear progenitor cells of myeloid hematopoietic origin (Figure 4A). Macrophages and dendritic cells important for immune function arise from the same bone marrow progenitor pool of cells (131). For osteoclastogenesis to occur, progenitor cells must be activated by macrophage colony-stimulating factor (M-CSF), which is needed for proliferation and survival of osteoclasts, and by receptor activator of nuclear factor-κB (RANK) ligand (RANKL), which is required for osteoclast differentiation. M-CSF is expressed by many different cells, including osteoblasts in the periosteum and stromal cells in the bone marrow. RANKL is expressed more restrictively and was initially thought to be produced only by osteoblasts/bone marrow stromal cells and by T lymphocytes and to be involved in osteoclastogenesis and activation of dendritic cells (132). A variety of hormones, cytokines, and prostaglandins [eg, PTH, $1,25(OH)_2$ D3, IL-1, IL-6, IL-17, TNF-α, and prostaglandin E2], which stimulate bone resorption, were initially reported to increase RANKL in periosteal osteoblasts; however, conditional deletion of *Rankl* in experimental studies has now suggested that expression of *Rankl* by osteocytes represents the most important source of RANKL for remodeling of the skeleton (133, 134).

Activation of the receptor RANK is dependent not only on the amount of RANKL present, but also on the amount of decoy receptor, osteoprotegerin (OPG), that is present. OPG is expressed ubiquitously and can bind RANKL, inhibiting binding to RANK. In addition to signaling through RANK and the c-Fms receptor for M-CSF, stimulation of the adapter proteins Fc receptor common γ-subunit (FcRγ) and DNAX activating protein of 12 kDa (DAP12), which are dimerized to Ig-like receptors such as osteoclast-associated receptor (OSCAR) and triggering receptor expressed on myeloid cells 2 (2), is required for stimulation of osteoclast differentiation (132). Genetic experiments have shown that mice overexpressing OPG, or with a deletion of RANKL, RANK, or c-Fms, or double knockout of FcRγ/DAP12, lack osteoclasts and exhibit osteopetrosis. Osteopetrosis is also observed in mice with a mutation in the gene encoding M-CSF. In contrast, mice lacking OPG exhibit early-onset osteoporosis (131, 132).

At a certain stage, mononuclear osteoclasts will fuse to latent multinucleated osteoclasts, which eventually will be activated to polarized bone-resorbing osteoclasts. The fusion process is not well understood, but dendritic cell-specific transmembrane protein (Dc-stamp) seems to be involved (135).

### C. RANK/c-Fms/FcRγ-DAP12 intracellular signaling

Intracellular signaling events downstream of RANK/c-Fms/FcRγ-DAP12 have been extensively investigated during the past decade (Figure 4B). The intracellular tail of RANK expresses several binding sites for TNF-related associated factors (TRAFs), of which TRAF6 seems to be most important (131, 132). Subsequent, proximal events include activation of several kinases, including MAPKs, inhibitor of nuclear factor-κB (NF-κB) kinase β (IKKβ), phosphoinositide 3-kinase (PI3K), and Akt. FcRγ/DAP12 signaling causes activation of phospholipase Cγ (PLCγ), a rise of intracellular calcium, and subsequent activation of the phosphatase calcineurin. Downstream events include activation of several transcription factors, such as NF-κB, c-Fos containing AP-1 (activator protein-1), nuclear factor of activated T cells c1 (Nfatc1), CREB, microphthalmia-associated transcription factor (MITF), and PU.1, which cooperate to regulate a multitude of genes (eg, *Calcr* [calcitonin receptor], *Acp5* [TRAP], *Ctsk* [cathepsin K], *Atp6i* [proton pump subunit], *αvβ3* [vitronectin receptor], *Clcn7* [chloride channel 7], and *Dc-stamp*) that are important for osteoclast differentiation, fusion, and function. In addition, several transcription factors, including v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MafB), interferon regulatory factor-8 (IRF-8), and B-cell lymphoma 6 (Bcl6), negatively regulate osteoclastogenesis (136), and the expression of these factors is repressed during osteoclast differentiation.

In vitro experiments have shown that mature osteoclasts can be formed from progenitor cells present in bone marrow, spleen, peripheral blood, and periosteum if M-CSF and RANKL are used as stimulators. However, the extent to which osteoclast-inducing hormones and cytokines affect progenitor cells in bone marrow, spleen, the circulation, or periosteum/endosteum is less clear, and it is not known whether there are differences in phenotypes of the progenitors from different sources. It has been reported that mature osteoclasts exhibit more phenotypic variation than realized previously, suggesting that progenitor cells from different sites might exhibit significant differences also (137).

**Figure 4.**

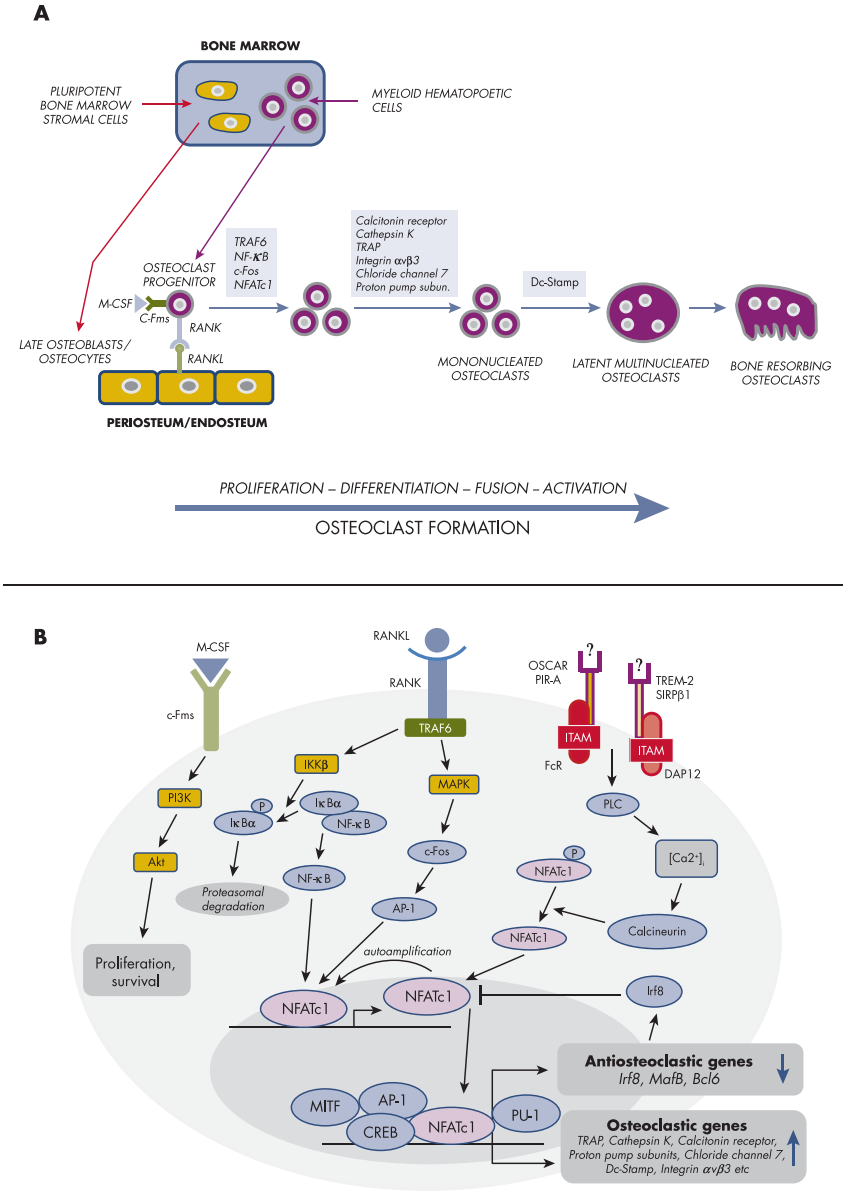


Figure 4. Extra- and intracellular regulation of osteoclast formation. A, Osteoblasts on the surfaces of cortical and trabecular bone originate from pluripotent stromal cells present in bone marrow. These cells not only make bone but also control the formation of osteoclasts. Osteoblasts control differentiation of mononuclear progenitor cells to mature, multinucleated osteoclasts by expressing M-CSF and RANKL, which expand the number of myeloid progenitor cells and promote their differentiation, respectively. Genes up-regulated at different stages of osteoclast differentiation are shown in squares. Mononucleated osteoclasts fuse to latent multinucleated osteoclasts, which subsequently become activated and attach to mineralized bone surfaces. Activated osteoclasts resorb bone by dissolving hydroxyapatite crystals and degrading extracellular bone matrix proteins. B, M-CSF activates its cognate receptor c-Fms, leading to activation of intracellular signaling like PI3K and Akt, which are important for proliferation and survival of the progenitor cells. RANKL activates its receptor RANK, which will recruit TRAF6 and subsequently activate several kinases and downstream transcription factors. In the cytosol, the dimeric transcription factor NF-κB is bound to its inhibitor, IκBα; activation of IKKβ by RANK signaling leads to phosphorylation of IκBα and dissociation from NF-κB, which then translocates to the nucleus and binds NF-κB response elements in DNA. Downstream signaling of RANK also involves activation of MAPK and phosphorylation of proteins like the c-Fos component of AP-1. These events are crucial for induction of Nfatc1, the master transcription factor of osteoclastogenesis. In its inactive form, Nfatc1 is phosphorylated, and activation is caused by dephosphorylation mediated by calcineurin, which is activated by signaling from FcRγ and/or DAP12 linked to Ig-like receptors expressed on the surface of osteoclasts. Nfatc1 acts in concert with other transcription factors like MITF, CREB, AP-1, and PU.1 to induce numerous genes necessary for osteoclast differentiation, fusion, and function. Osteoclast differentiation also requires down-regulation of genes such as Irf8, MafB, and Bcl6 associated with the macrophage phenotype. ITAM, immunoreceptor tyrosine-based activation motif. TREM-2, triggering receptor expressed on myeloid cells 2.

## VIII. Retinoids, Osteoclast Differentiation, and Bone Resorption in Organ-Cultured Intact Bones

During the 1920s, animal experiments established that excess vitamin A had profound effects on the skeleton, including thinning of long bones and spontaneous fracture. In 1934, Strauss and Maddock (138) noted more osteoclasts ("osteoclasis") associated with hypervitaminosis A, but it was not known at the time whether the skeletal effects associated with vitamin A in intact animals were indirect or direct. Barnicot (139) was the first to obtain evidence that vitamin A might directly affect bone. He noted erosion of bones after adding fragments of crystalline vitamin A acetate to parietal bones from newborn mice and transplanting them to cerebral hemispheres of littermates (139). Subsequent, more convincing evidence that vitamin A directly affected bone was presented by Fell and Mellanby (140) at Strangeways Laboratories in Cambridge, United Kingdom. In their experiments, plasma with vitamin A added or plasma from fowl treated with high doses of vitamin A was added to explant cultures of either chicken embryonic limb buds obtained from eggs or to fetal mouse long bones. Excess vitamin A led to shrinkage of cartilage, and the bone nearly disappeared; the periosteal bone had a "moth-eaten appearance." Despite the fact that bone was diminished in all the explants, the number of osteoclasts was only increased in some of the explants, and the authors speculated that macrophages, endothelial cells, reticular cells, or possibly osteoblasts/osteocytes might have been responsible for the actions of vitamin A.

In later experiments, Raisz (141) used fetal rat long bones cultured in medium with a high (50%) content of serum and found that vitamin A stimulated resorption and increased the number of osteoclasts in cultures. It was noted that the number of osteoclasts formed in vitamin A-stimulated bones was fewer than those formed in PTH-stimulated bones, suggesting that the 2 compounds caused bone breakdown by different mechanisms. At the time of Raisz' experiments, vitamin A had been reported to release lysosomal enzymes from isolated lysosomes, and it was suggested that this might explain the differences between vitamin A and PTH on bone breakdown.

Clinical studies have used primarily retinol or retinyl esters to determine vitamin A status; however, in ex vivo and in vitro studies discussed in *Sections VIII* and *IX*, the active metabolite ATRA is normally used to study effects of retinoids. The in vivo physiological level of ATRA in human serum is approximately 2–20 nmol/L (150-fold lower than retinol) (142). Pharmacological levels of ATRA can exceed 100 nM in serum (143). Serum levels of ATRA are somewhat higher in humans than in mice and rats (144, 145). The tissue concentrations of ATRA are generally higher than serum concentrations because ATRA is produced locally. Mouse liver, kidney, brain, and testis all have higher ATRA concentrations than serum (7–11 pmol/g tissue compared to approximately 2 pmol/g serum) (145). Concentrations can vary significantly over short distances; eg, ATRA levels of 0.6 nmol/L have been reported in the upper zones of the growth plate, compared to about 1.8 nmol/L in the lower parts of the plate, whereas the perichondrium was found to be quite rich in ATRA (about 4.9 nmol/L) (33). Interestingly, the levels of retinol were found to be similar in all 3 locations (1.1–1.6 mmol/L). Increased ATRA levels have also been reported in synovial fluid from patients suffering from osteoarthri-

**Table 2.** Summary of the Outcome of ex Vivo Studies Investigating the Effect of Retinoids on Bone Resorption and Osteoclast Formation

| Bone Specimens | Retinoid | Concentration | Resorption | Osteoclast | Ref. |
|---|---|---|---|---|---|
| Fetal rat radius/ulna | Vitamin A | 10 $\mu$g/mL | ↑ | (↑) | 141 |
| Newborn mouse calvaria | Retinol | $10^{-5}$ mol/liter | ↑ | ? | 148 |
| Fetal mouse calvaria | ATRA | $10^{-6}$ mol/liter | ↑ | ? | 149 |
| Newborn mouse calvaria | ATRA | $10^{-8}$ to $10^{-6}$ mol/liter | ↑ | ? | 150 |
| Newborn mouse calvaria | ATRA | $3 \times 10^{-10}$ to $3 \times 10^{-7}$ mol/liter | ↑ | ? | 151 |
| | 9-*cis* RA | $3 \times 10^{-10}$ to $3 \times 10^{-7}$ mol/liter | ↑ | ? | |
| Newborn mouse calvaria | ATRA | $10^{-8}$ mol/liter | ↑ | ? | 152 |
| Fetal rat calvaria | Retinol | $2 \times 10^{-6}$ mol/liter | ↑ | ? | 153 |
| Fetal rat tibiae | ATRA | $10^{-6}$ mol/liter | ↑ | ↑ | 154 |
| Fetal rat metatarsals | ATRA | $10^{-6}$ mol/liter | ↑ | ↑ | 154 |
| Newborn mouse calvaria | ATRA | $10^{-10}$ to $10^{-7}$ mol/liter | ↑ | ↑ | 89 |
| | 9-*cis* RA | $10^{-9}$ to $10^{-6}$ mol/liter | ↑ | ? | |
| | Retinol | $10^{-8}$ to $10^{-6}$ mol/liter | ↑ | ? | |
| | TTNPB | $10^{-10}$ to $10^{-7}$ mol/liter | ↑ | ? | |
| | GR104 | $10^{-10}$ to $10^{-7}$ mol/liter | ↑ | ? | |

↑, increase; (↑), minor increase; ?, not investigated.

**Table 3.** Summary of the Outcome of in Vitro Studies Investigating the Effect of Retinoids on Osteoclast Formation

| Osteoclast Progenitor Cells | Stimulator[a] | Retinoid | Concentration of Retinoid | Osteoclast[b] | Ref. |
|---|---|---|---|---|---|
| Rat nonadherent bone marrow cells | — | ATRA | $10^{-6}$ mol/liter | — | 154 |
| Human mononuclear bone marrow cells | — | ATRA | $10^{-6}$ mol/liter | — | 156 |
| Egg-laying hen mononuclear bone marrow cells | — | ATRA | $10^{-6}$ mol/liter | ↑ | 159 |
| Mouse bone marrow cells + mouse calvarial cells | — | ATRA | $10^{-9}$ to $10^{-6}$ mol/liter | — | 158 |
| Mouse bone marrow cells | — | ATRA, 9-*cis* RA | $10^{-7}$ mol/liter | — | 155 |
| Rat bone marrow cells | D3 | ATRA | $10^{-6}$ mol/liter | ↓ | 157 |
| Mouse bone marrow cells + mouse calvarial cells | D3 | ATRA | $10^{-9}$ to $10^{-6}$ mol/liter | ↓ | 158 |
| Mouse bone marrow cells | D3 | ATRA,9-*cis* RA | $10^{-7}$ mol/liter | ↓ | 155 |
| | PTH | ATRA | $10^{-7}$ mol/liter | ↓ | |
| | RANKL | ATRA | $10^{-7}$ mol/liter | ↓ | |
| Mouse nonadherent bone marrow cells | RANKL | ATRA | $10^{-8}$ to $10^{-7}$ mol/liter | ↓ | 75 |
| Mouse spleen cells | RANKL | ATRA | $10^{-7}$ mol/liter | ↓ | 155 |
| Mouse bone marrow macrophages | RANKL | ATRA | $10^{-10}$ to $10^{-7}$ mol/liter | ↓ | 155 |
| | | GR104 | $10^{-10}$ to $10^{-7}$ mol/liter | ↓ | |
| | | 9-*cis* RA | $10^{-7}$ mol/liter | ↓ | |
| | | 13-*cis*RA | $10^{-7}$ mol/liter | ↓ | |
| | | TTNPB | $10^{-7}$ mol/liter | ↓ | |
| Human $CD14^+$ monocytes | RANKL | ATRA | $4 \times 10^{-11}$ to $10^{-6}$ mol/liter | ↓ | 160 |

Abbreviations: D3, $1,25(OH)_2$ D3; ↓, decrease; ↑ increase; —, no effect.

[a] Osteoclast formation was stimulated by well-known agents when assessing the effect by retinoids.

[b] Number of multinucleated $TRAP^+$ cells was counted to quantify osteoclast formation.

tis (146). The retinol concentration in total extracts of femur and tibia from normal rats has been observed to be approximately 500–600 nmol/g and to increase after a diet high in vitamin A (147). Local concentrations of ATRA in different bone compartments have not been investigated as thoroughly.

Tables 2, 3, and 4 show concentrations of the different retinoids that have been used in the ex vivo and in vitro studies summarized in *Sections VIII* and *IX*. It appears that several studies have been performed using concentrations of ATRA in the micromolar range, which is clearly much higher than physiological concentrations and most likely higher than those obtained during hypervitaminosis A; however, most of the biological effects reported by such high concentrations of ATRA have also been observed in other studies at lower concentrations (1–10 nM).

### A. Direct stimulation of bone resorption by vitamin A

The first evidence for a direct stimulatory effect by vitamin A on bone resorption in chemically defined medium with no serum, or low serum (5%), was provided by Raisz et al (148) in 1977 when it was demonstrated that retinol stimulated radioactive calcium release from newborn mouse parietal bones. It was also shown that retinol had no effect in osteopetrotic *mi/mi* mice (unable to form osteoclasts due to mutations in the *Mitf* gene), demonstrating that osteoclasts are necessary for the bone-resorptive effect of vitamin A. Since then, additional publications have shown that retinol or ATRA can stimulate bone resorption in fetal (149) or neonatal mouse calvariae (150–152), fetal rat calvariae (153), or fetal rat tibiae or metatarsals (154), and that the resorptive effect of vitamin A can be inhibited by bisphosphonates (150).

Recently, it was demonstrated that ATRA stimulates mineral release and bone matrix degradation in neonatal mouse calvarial bones and that osteoclast inhibitors such as zoledronic acid and calcitonin blocked the bone-resorptive effects of ATRA (89). ATRA was also observed to stimulate the number of cathepsin K-positive multinucleated osteoclasts and increase the mRNA expression of

**Table 4.** Summary of the Outcome of in Vitro Studies Investigating the Effect of Retinoids on Osteoclast Activity

| Mature Osteoclasts | Retinoid | Concentration | Osteoclast Activity[a] | Ref. |
|---|---|---|---|---|
| Rabbit bone marrow osteoclasts on dentine | ATRA | $10^{-12}$ to $10^{-9}$ mol/liter | ↑ | 161 |
| Rabbit bone marrow osteoclasts on bone | 13-*cis* RA | $10^{-6}$ mol/liter | ↑ | 162 |
| Chicken bone marrow osteoclasts on either bone or dentine | ATRA | $10^{-8}$ to $10^{-6}$ mol/liter | ↓ | 163 |

↓, decrease; ↑, increase.

[a] Osteoclast formation was assessed as number or area of pits formed in bone or dentine slices.

genes encoding calcitonin receptor, TRAP, and cathepsin K, showing that increased osteoclast differentiation and formation are important for the bone-resorptive effect of vitamin A; however, these studies did not reveal whether retinoids can affect mature osteoclast activity.

The evidence from different ex vivo bone cultures indicates that vitamin A has the capacity to stimulate bone resorption (Table 2).

#### B. Vitamin A stimulates bone resorption through increased RANKL mediated by RARα

In cultured calvarial bones, ATRA was additionally found to robustly increase the mRNA and protein expression of RANKL, with no effect on *Rank* mRNA expression (89). Furthermore, ATRA caused a transient decrease of *Opg* mRNA with no effect on OPG protein. These data indicate that ATRA causes increased bone resorption primarily by enhancing RANKL. This idea is supported by experiments showing that OPG added exogenously to ATRA-stimulated bones abolishes the bone-resorptive effect and the increased mRNA expression of *Ctsk* stimulated by ATRA. It remains, however, to be shown which cell type it is in bone that responds to ATRA with increased expression of RANKL.

Experiments using a variety of different agonists for RARα, RARβ, and RARγ have additionally suggested that the bone-resorptive effect of ATRA in neonatal mouse calvariae and stimulation of RANKL mRNA and protein are due to activation of RARα (89). Although conclusive evidence for this observation must await studies using cells from knockout mice or cells in which the receptors have been knocked down with silencing interference RNA, it has also been shown that stimulation by ATRA is concentration-dependently inhibited by an RARα antagonist, further implicating RARα as the important receptor subtype for stimulation of RANKL and periosteal resorption in cortical bone.

It has been reported that ATRA may have proinflammatory effects. Thus, the possibility exists that ATRA might increase RANKL and bone resorption by indirect mechanisms, perhaps mediated by cytokines capable of stimulating RANKL and bone resorption. In recent experiments, however, no evidence has been found for ATRA to affect mRNA expression of *Il1β*, *Il6*, or *Tnfα* in calvarial bones or of *Cox2* mRNA being affected (89).

## IX. Retinoids and Osteoclast Differentiation and Activity in Cell Cultures

Experiments with organ-cultured bones do not reveal how osteoclastogenic agents such as retinoids affect osteoclast formation and function. These questions can best be answered by performing cell culture experiments employing osteoclast progenitors from bone marrow, spleen, or peripheral blood. Osteoclast progenitors are stimulated with M-CSF and RANKL or incubated with bone marrow stromal cells or periosteal osteoblasts in the presence of hormones or cytokines that stimulate RANKL in the supporting cells. The data obtained in cell cultures (Table 3) assessing different effects by retinoids on osteoclast formation are in several aspects contrasting to those obtained in ex vivo bone organ cultures. An attempt to reconcile data generated ex vivo/in vitro with those obtained in vivo is presented in *Section X*.

#### A. Inhibition of osteoclast progenitor cells by retinoids

Most studies to assess the effect of retinoids on osteoclastogenesis have been performed using either bone marrow cultures stimulated by $1,25(OH)_2$ D3 or purified osteoclast progenitors stimulated by M-CSF and RANKL. In recent experiments using mouse bone marrow cultures containing supporting stromal cells and hematopoietic cells, it has been shown that PTH and $1,25(OH)_2$ D3 can stimulate formation of $TRAP^+$ multinucleated osteoclasts capable of resorbing bone; however, no osteoclasts were observed in parallel cultures stimulated by ATRA or 9-*cis* RA (155). In contrast to $1,25(OH)_2$ D3, ATRA did not induce expression of genes associated with osteoclast differentiation, such as those encoding the calcitonin receptor, TRAP, cathepsin K, Nfatc1, or c-Fos, indicating that ATRA was not able to initiate differentiation of osteoclast progenitor cells. This was explained by showing that ATRA, unlike $1,25(OH)_2$ D3, did not induce the mRNA expression of *Rankl*. It was also observed that $1,25(OH)_2$ D3, but not ATRA, can stimulate *Rankl* mRNA in the mouse ST-2 stromal cell line (our unpublished observations).

In a study by Thavarajah et al (156), where gradient centrifugation was used to purify mononuclear cells from human rib bone marrow, no effect of ATRA was reported after 3–4 weeks of culture, but enhanced osteoclastogenesis was noted with $1,25(OH)_2$ D3. These results are very similar to the observations with ATRA and $1,25(OH)_2$ D3 in mouse bone marrow cultures and suggest that the purified mononucleated cells used in the study by Thavarajah et al (156) contained stromal cells. Similarly, Scheven and Hamilton (154), using nonadherent mononuclear cells from rat bone marrow, found no effect of ATRA, but stimulation of osteoclastogenesis with $1,25(OH)_2$ D3, again suggesting contamination with stromal cells. Thus, whereas ATRA can stimulate RANKL and periosteal osteoclast formation in mouse organ cultures of parietal bones, in bone marrow cultures that are dependent on

stromal cells for RANKL formation and osteoclastogenesis, evidence indicates that ATRA is ineffective.

Several studies have suggested that retinoids can directly inhibit osteoclast progenitor cells in bone marrow and spleen. Hata et al (157) first reported that ATRA inhibited formation of osteoclasts in rat bone marrow cell cultures stimulated by $1,25(OH)_2$ D3. Later, Wang et al (158) used a coculture system containing mouse bone marrow cells to which primary calvarial osteoblasts from newborn mice were added, and they reported that ATRA ($10^{-6}$ to $10^{-9}$ M) inhibited osteoclast formation induced by $1,25(OH)_2$ D3. Similarly, the synthetic acyclic retinoid, geranylgeranoic acid, also inhibited osteoclast formation stimulated by $1,25(OH)_2$ D3, although with less potency than ATRA. In agreement with these observations, it has been observed that osteoclast formation stimulated by either $1,25(OH)_2$ D3 or PTH in bone marrow cultures containing stromal cells (with no osteoblasts added) is potently inhibited by both ATRA and 9-*cis* RA (155). Inhibition was associated with decreased mRNA expression of osteoclastic genes, with no effect on $1,25(OH)_2$ D3-induced up-regulation of *Rankl* mRNA or down-regulation of *Opg* mRNA, suggesting that osteoclast progenitors, rather than stromal cells, were targeted by the retinoids to cause inhibition. In support of this, studies have also shown that stimulation of osteoclast formation in mouse spleen cell cultures induced by M-CSF/RANKL is inhibited by ATRA and 9-*cis* RA (155); however, in both the mouse bone marrow and spleen cell cultures, it could not be completely ruled out that retinoids inhibited osteoclast differentiation by some factor(s) released from other cells contaminating the crude cell cultures.

In contrast to observations suggesting that retinoids inhibit differentiation of osteoclast progenitor cells, Chiba et al (159) reported that formation of $TRAP^+$ multinucleated osteoclasts occurred after $10^{-6}$ M ATRA treatment of purified nonadherent bone marrow mononuclear cells from egg-laying hens fed a calcium-deficient diet for 2–3 weeks. When these cells were incubated with $^3H$-labeled bone particles, the osteoclasts were incapable of degrading the particles. The authors concluded that ATRA stimulates formation of osteoclasts lacking bone-resorbing activity. No comparison with a compound like $1,25(OH)_2$ D3 was performed, so it is not possible to judge whether the chicken cultures were free of contaminating cells, such as stromal cells. Furthermore, the reliability of the bone particle assay for use in measuring resorptive activity of mature osteoclasts has not been demonstrated.

In attempts to show that osteoclast progenitors are the target cells for the inhibition caused by retinoids and to study mechanisms of retinoid-induced inhibition of osteoclast formation, mouse bone marrow macrophages have been prepared by incubating bone marrow cells on plastic dishes to which stromal cells cannot adhere, expanding the number of macrophages with M-CSF for 2–3 days, then using only cells that adhere to the plastic dishes for study. No stromal cells are present with adherent cells in these preparations, the adherent cells do not form osteoclasts when treated with $1,25(OH)_2$ D3 or PTH, and amplification of *Akp1* (gene encoding alkaline phosphatase) and *Rankl* transcripts does not occur. ATRA cannot stimulate osteoclast formation in such purified bone marrow macrophages, but when the cells are stimulated with RANKL, ATRA abolishes osteoclast formation, with half maximal inhibition occurring at 0.3 nM (155). The most potent inhibition occurred when ATRA was added with RANKL to the cultures; when ATRA was added 24 or 48 hours after RANKL, inhibition gradually decreased. These observations suggest that the inhibitory action of ATRA occurs at an early stage of osteoclast differentiation. In support of this idea, it was observed that withdrawal of ATRA from culture medium 6 hours after initial treatment with RANKL plus ATRA still caused profound inhibition of osteoclastogenesis. Furthermore, it was determined that inhibition with ATRA was not due to cell toxicity or inhibition of cell proliferation. ATRA was also found to be a potent inhibitor of osteoclast formation when spleen cells were used as osteoclast precursors. Moreover, mRNA expressions of genes associated with osteoclast differentiation (eg, *Calcr*, *Ctsk*, and *Acp5*) were strongly inhibited by ATRA, with half maximal inhibition at 0.3 nM. Up-regulation of mRNA expression for the macrophage transcription factor *MafB* also occurred with ATRA treatment, indicating that differentiation of bone marrow macrophages to osteoclasts is decreased. In agreement with these observations, Kneissel et al have shown that ATRA can inhibit osteoclast formation in cultures of nonadherent monocytes/macrophages from adult mice treated with RANKL (75).

It has been reported recently that ATRA can also inhibit formation of mature osteoclasts in cultures of purified $CD14^+$ monocytes stimulated with RANKL (160). Stimulation of total TRAP activity by RANKL was abolished by ATRA concentrations at and above 0.04 nM. In a manner similar to the effect noted with ATRA in mouse bone marrow macrophages, ATRA seemed to arrest the $CD14^+$ osteoclast progenitor cells at a monocyte/macrophage state, for mRNA of the macrophage transcription factor *Irf-8* was increased by ATRA in $CD14^+$ cells. Interestingly, the authors reported that ATRA could enhance the number of M-CSF-stimulated $CD14^+$ cells in culture. Whether or not this effect was due to increased prolifer-

ation, decreased apoptosis, or a combination of both was not investigated. How this observation is linked to regulation of osteoclast formation by ATRA remains to be shown.

### B. RANK signaling is inhibited by retinoids in osteoclast progenitor cells

To assess which signal-transducing mechanisms might be affected by ATRA, the important osteoclast mediators AP-1, NF-κB, and Nfatc1 have been evaluated (155). ATRA was observed to inhibit (half maximal inhibition at approximately 0.3 nM) the increased mRNA and protein expressions of *cFos* and *Nfatc1* stimulated by RANKL in purified bone marrow macrophages. Decreased mRNA expressions of the NF-κB inhibitor, inhibitory κBα (*IκBα*), and of the NF-κB subunits, *p52* and *RelB*, stimulated by RANKL were also noted after ATRA exposure, indicating that the NF-κB pathway is inhibited by ATRA as well. The inhibition of AP-1, Nfatc1, and NF-κB expression by ATRA provides a good explanation for the inhibition of osteoclast differentiation by the retinoid.

Osteoclast differentiation requires stimulation of the receptors RANK and c-Fms by RANKL and M-CSF, respectively. ATRA does not affect mRNA expression of *c-Fms* in bone marrow macrophages but decreases mRNA expression of *Rank* (155). Inhibition of *Rank* mRNA is a delayed response and is not observed until 24 to 48 hours after exposure to ATRA. In contrast, inhibition of *c-Fos* and *Nfatc1* mRNA can be observed within 6 hours after ATRA exposure. This early inhibition suggests that ATRA inhibits osteoclastogenesis primarily by interfering with signal transduction pathways downstream of RANK, rather than by inhibiting RANK expression. In support of this, it has been shown recently that ATRA can inhibit RANKL-induced osteoclast differentiation in mouse macrophages transfected with a lentivirus containing the *Rank* gene and overexpressing RANK (our unpublished observations).

As mentioned in *Section IX.A.*, ATRA also inhibits osteoclast formation in RANKL-stimulated cultures of human $CD14^+$ monocytes (160). When osteoclast differentiation in these experiments was assessed by analyzing the mRNA expression of *Nfatc1*, significant inhibition by ATRA was observed when cells were cultured on plastic, but not when cells were cultured on bone, although osteoclast formation was abolished both on bone and plastic, and inhibition of *Ctsk* mRNA was seen in both instances. Furthermore, it was reported that *Rank* mRNA was decreased by ATRA when RANKL-stimulated $CD14^+$ cells were cultured on bone, but that no significant effect was noted when cells were cultured on plastic, although RANK protein was decreased on plastic. In contrast to mouse bone marrow macrophages, RANK mRNA and protein were not up-regulated by RANKL in the $CD14^+$ human monocytes.

### C. RARα receptors involved in inhibition of osteoclast progenitors

Bone marrow macrophages exhibit abundant expression of *Rarα* mRNA, but less expression of *Rarβ* and *Rarγ* mRNA (155). Based on fluorescence-activated cell sorting analysis, most purified macrophages express intracellular RARα and RARβ protein, but very few of the cells express RARγ protein. To evaluate which of the RARs were required for inhibition of osteoclast differentiation by ATRA, purified macrophages were incubated with RANKL with or without different retinoids. Inhibition of osteoclast formation and mRNA expression of *Calcr*, *c-Fos*, and *Nfatc1* were found with retinoids that are recognized by the 3 RARs: ATRA, 9-*cis* RA, 13-*cis* RA, and TTNPB (4-[(*E*)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid). Inhibition equal to that seen with ATRA was noted with an RARα specific agonist, GR104. The RARα antagonist, GR110, reversed inhibition by ATRA. These experiments suggest that RARα is responsible for the inhibition of osteoclastogenesis stimulated by RANKL. In support of this, it has been shown recently that knockdown of *Rarα* mRNA in bone marrow macrophages by interference silencing substantially decreases the effect of ATRA to inhibit RANKL-induced osteoclastogenesis (our unpublished observations). Experiments with A7980 and GR103, which have affinities for RARγ and RARβ/γ, respectively, have also indicated that both compounds are significantly less potent inhibitors of osteoclastogenesis stimulated by RANKL than either ATRA or GR104. These data suggest that RARβ and RARγ are not as important as RARα for inhibition of osteoclastogenesis stimulated by RANKL, but conclusive evidence for this awaits studies where RARβ and RARγ are knocked down by interference silencing or use of cells from mice in which these receptors have been deleted.

As discussed previously, ATRA can bind intracellularly to FABP5 and be shuttled to PPARβ/δ (39). Bone marrow macrophages express *Fabp5* mRNA, and the possibility that ATRA might inhibit osteoclast differentiation by activation of PPARβ/δ was evaluated. It was found that the PPARβ/δ agonist, GW072, did not inhibit osteoclastogenesis stimulated by RANKL, but rather potentiated the formation of numerous, extremely large osteoclasts in the presence of RANKL (155).

Experiments employing different RAR agonists and antagonists have also demonstrated that inhibition of

osteoclast formation in RANKL-stimulated human $CD14^+$ cells is mediated by RAR$\alpha$ (our unpublished observations).

Although data from ex vivo studies indicate that ATRA up-regulates RANKL and causes significant stimulation of mouse periosteal bone resorption, it has also been observed that ATRA exerts potent inhibition of RANKL-induced differentiation in mouse bone marrow, spleen cell, and human blood osteoclast progenitor cells. Why periosteal osteoclast progenitor cells are not inhibited by ATRA is unclear. One possibility is that osteoclast progenitor cells in the periosteum are different from those in the bone marrow and circulation. When bone resorption in mouse calvarial bones is stimulated by RANKL together with ATRA, inhibition of RANKL-induced osteoclast formation does not occur (155), so it seems possible that progenitor cells in the periosteum might lack retinoid acid receptors, or perhaps periosteal cells might release molecules making the osteoclast progenitor cells resistant to ATRA, but answers to these and other questions await further experimentation.

#### D. Effects of retinoids on mature osteoclasts

Divergent results have been obtained when the effects of retinoids have been assessed using mature osteoclasts (Table 4). Increased pit number and increased mRNA expression of *Ctsk* have been observed after ATRA ($\geq$1 pM) treatment of rabbit osteoclasts cultured on dentin slices (161). Increased numbers of resorption pits accompanied by altered microfilament morphology have also been demonstrated with the retinoid isotretinoin (13-*cis* RA; 1 $\mu$M) in rat osteoclasts incubated on bovine cortical bone slices (162). In contrast, ATRA ($\geq$10 nM) has been reported to inhibit the bone-resorbing activity of embryonic chicken osteoclasts incubated on either bovine cortical bone slices or sperm whale dentin (163). Bone-resorbing activity of mature osteoclasts has also been studied by incubating osteoclasts with bone particles instead of bone slices; however, degradation of bone in the bone particle assay may be more a reflection of phagocytosis by osteoclasts than true bone resorption (ie, formation of ruffled border, resorption lacunae, etc). When the bone particle assay has been employed for study, ATRA has been observed to stimulate chicken osteoclast activity but to inhibit the activity of osteoclast-like, human, giant bone tumor cells (164). Finally, activation by ATRA (1 $\mu$M) of mature osteoclasts from egg-laying hens, based on expression of integrin $\alpha_v\beta_3$ and activation of latent TGF$\beta$, has been reported (165). Although these studies suggest that RARs are present in mature osteoclasts, it is not possible at this time to determine how the receptors might be regulating the bone-resorbing activity of the mature osteoclasts.

## X. Retinoids and in Vivo Bone Resorption

As mentioned previously, it has been known for nearly a century that hypervitaminosis A causes increased bone fragility and fractures in experimental animals. Trechsel et al (166) showed that treatment of thyroparathyroidectomized adult male rats with the retinoid, Ro 13–6298 (25 $\mu$g sc for 2–4 d), resulted in hypercalcemia, increased release of calcium from bones, and decreased bone mass. Treatment with different bisphosphonates was observed to attenuate the decrease in bone mass, suggesting that the effects of Ro 13–6298 were due to enhanced bone resorption. In support of this idea, it was observed that large numbers of mature osteoclasts were present at subperiosteal sites of bone; however, only a few subsequent studies in animals have attempted to examine in detail how vitamin A affects different parts of the skeleton and why bones become fragile.

Hough et al (153) reported that hypervitaminosis A in male rats (10 000 to 25 000 IU vitamin A administered as retinyl palmitate by stomach tube daily for 21 d) causes increased bone resorption, increased numbers of tibial osteoclasts, and increased urinary hydroxyproline, while having no effect on plasma calcium or circulating levels of PTH or 25(OH) D.

Kneissel et al (75) performed an extensive study on female rats given the retinoid Ro 13–6298 sc (125 $\mu$g/kg) for 4 days. In proximal tibiae, bone mineral content and area of cortical bone were substantially reduced in both 12- and 56-week-old rats. No effects were noted in trabecular bone. Retinoid treatment increased circulating TRAP, suggesting that the bone loss was due to increased bone resorption. This idea was supported by the observation that the decrease in bone mass was reduced by the bisphosphonate, alendronate. Similar observations were made in female rats treated with 120 mg/kg ATRA orally for 4 days. Furthermore, 15-week-old male mice treated with Ro 13–6298 sc (125 $\mu$g/kg) for 4 days exhibited bone loss in the proximal tibia and distal femur that was attributed totally to loss of cortical bone. Histological studies in rats showed that osteoclast surface area and numbers at periosteal sites were enhanced by retinoid treatment, which explains the loss of cortical bone and increased circulating TRAP, as well as alendronate blocking bone loss. In contrast, reduced numbers of osteoclasts were found on trabecular bone, which explains why bone loss was not observed there. No data on bone formation at trabecular sites were provided, but it seems reasonable to assume that

bone formation may have been decreased in trabecular bone because bone mass was not increased, despite the absence of trabecular osteoclasts. These studies have shown that increased periosteal bone resorption is a rapid response to hypervitaminosis A. The studies also demonstrate a remarkable heterogeneity in the cortical and trabecular responses to vitamin A in long bones. Whether or not vertebral bones or flat bones are as sensitive to vitamin A is not known currently.

The in vivo data noting stimulation of cortical bone resorption by retinoids are in good agreement with in vitro observations showing increased periosteal bone resorption in organ-cultured bone. There also appears to be good agreement between in vivo data indicating that retinoids reduce the numbers of osteoclasts on trabecular bone with in vitro observations showing inhibition of osteoclast differentiation in crude bone marrow cells and purified bone marrow macrophages by retinoid treatment. These results tend to suggest that trabecular osteoclasts may be dependent on differentiation of progenitor cells from the bone marrow, whereas periosteal osteoclasts may originate from some other source.

More recently, Lind et al (76) investigated the effects of hypervitaminosis A on the skeleton of 5-week-old male rats. Over a period of 7 days, control rats were fed with pellets containing 12 IU vitamin A/g pellet, and experimental rats were given 1700 IU vitamin A/g pellet as a mixture of retinyl palmitate and retinyl acetate. In the femur, total bone mineral content and density were significantly reduced, resulting in weaker bones, as assessed by 3-point bending. Hypervitaminosis A decreased bone mineral content and density not only in cortical bone but also in trabecular bone (76). In addition, circulating levels of TRAP5b and carboxy-terminal collagen crosslinks were decreased, suggesting inhibition of bone resorption; supporting this view, serum RANKL was decreased with no change in OPG. In accordance with several previous studies, an enhanced number of osteoclasts was observed at periosteal surfaces, but no change in osteoclast numbers was noted for trabecular bone. Increased periosteal resorption offers an explanation for the decrease in cortical bone, but increased resorption does not appear to explain the loss of trabecular bone or the decreases in serum carboxy-terminal collagen crosslinks and Trap5b. However, there was a drastic reduction in the number of mature osteoclasts at endosteal surfaces, and it was suggested that 8 days of hypervitaminosis A kills endosteal osteoclasts because of hypoxia caused by reduction of blood vessels in the bone marrow close to endosteal surfaces. This suggestion was supported not only by morphological observations but also by increased expression of hypoxia-related genes like *Hifα* and downstream genes such as *Twist1* and *Mmp2* in bone marrow from humeri in rats fed with pellets containing high concentrations of retinyl palmitate and retinyl acetate for 8 days (76). Similarly, a microarray analysis of the samples showed increased expression of other hypoxia-related genes such as *Entpd3*, *Nt5e*, *Bhlhe41*, and *Loxl2* (167). Observations in patients with APL support the findings by Lind et al (76). Like many malignant tumors, APL is associated with increased angiogenesis most likely due to vascular endothelial growth factor produced by the tumor cells. Interestingly, retinoid treatment of APL patients results in reduced numbers of bone marrow microvessels, an effect associated with decreased expression of vascular endothelial growth factor in the tumor cells (168).

The observations in the studies by Kneissel et al (75) and Lind et al (76) both show decreased cortical bone and increased formation of periosteal osteoclasts, which is in agreement with several previous studies; however, Kneissel et al (75) found no change in trabecular bone density although the number of osteoclasts was decreased, whereas Lind et al (76) found that trabecular bone was decreased but there was no change in the number of osteoclasts. Furthermore, the circulating levels of the resorption marker TRAP were different in the 2 studies, with increased levels observed by Kneissel et al (75) and decreased levels found by Lind et al (76). As regards the very interesting observation of decreased numbers of osteoclasts at the endosteal surfaces of cortical bone reported by Lind et al (76), no such observation was reported by Kneissel et al (75).

In a study by Wray et al (147), rats were fed after weaning with a diet supplemented with retinyl palmitate at 3 different doses. Effects on bone were assessed in young (2–3 mo), middle-aged (8–10 mo), and old (18–20 mo) rats. Supplementation was intended to result in reduced, normal, and increased levels of circulating vitamin A. It was observed that the medullary area was increased and cortical thickness decreased in young rats, but no such effects could be seen in middle-aged rats. In old rats, medullary area was decreased, an effect associated with decreased endosteal and periosteal radius. The study did not include any assessments at the cellular level, but the effects in old rats could be explained by observations made by others showing increased periosteal resorption (75, 76) and endosteal new bone formation (76). In agreement with Lind et al (76), increasing vitamin A was associated with decreased trabecular BMD. However, the changes in cortical and trabecular bone did not affect the resistance to fractures.

Although it seems clear that increased osteoclastogenesis and resorption of cortical bone at the periosteal sur-

face is an important mechanism leading to bone fragility in hypervitaminosis A (Table 5), additional experiments are needed to determine how vitamin A affects osteoclasts on trabecular bone and at endosteal surfaces.

## XI. Retinoids and Bone Formation

Increased bone fragility can be a consequence not only of increased bone resorption, but also of decreased bone formation. There are fewer studies on the effects of vitamin A on bone formation than on resorption. Several in vitro studies have suggested that retinoids inhibit adipocyte differentiation and stimulate osteoblast differentiation, as well as work with bone morphogenetic protein (BMP) to stimulate osteogenesis. In the preadipocyte cell line 3T3-F442A, ATRA has been shown to act synergistically with BMP-2 to inhibit insulin-induced adipocyte differentiation, as assessed by Oil Red O staining of lipids and mRNA expression of PPAR$\gamma$ (169). ATRA and BMP-2 have also been found to act synergistically to stimulate alkaline phosphatase activity in 3T3-F442A cells. Stimulation of mineralization and expression of the osteoblastic genes *Cbfa1/Runx2*, *Osteocalcin*, and *Collagen type I* by ATRA (0.001–1 $\mu$M) was also shown to be increased by overexpression of the BMP receptor II (169). Similarly, ATRA (2.5 $\mu$M) has been noted to stimulate alkaline phosphatase activity, *Osteocalcin* mRNA, and mineralization in the preadipocyte cell line 3T3-L1 (170, 171). Cotreatment with BMP-2 resulted in synergistic effects due to up-regulation of the BMP receptor IB by ATRA. It has also been reported that ATRA and 9-*cis* RA (5–20 $\mu$M) can stimulate alkaline phosphatase activity and osteocalcin mRNA and protein in the mesenchymal progenitor cell line C3H10T1/2, but that the compounds are not able to induce mineralization (171). On the other hand, in C3H10T1/2 cells overexpressing BMP-9, both retinoids have been observed to synergistically potentiate alkaline phosphatase, as well as late mineralization.

These data suggest that retinoids can induce osteoblast differentiation, but not bone formation; however, in concert with BMPs, they can stimulate both osteoblast differentiation and bone formation. Information on the effects of vitamin A on bone formation in vitro is still very sparse, and it is not possible to reach a firm conclusion regarding vitamin A action at this time.

In contrast to the in vitro studies described above, it has been reported that ATRA (1 $\mu$M) can inhibit alkaline phosphatase activity and mineralization in primary cultures of rat bone marrow stromal cells, a response associated with increased adipocyte differentiation (172). In the mouse calvarial osteoblastic cell line MC3T3-E1, no effects on alkaline phosphatase activity or BMP-2-induced enzyme activity were noted with 1 to 100 nM of the retinoid, Ro 13–6298 (75), but because a high concentration of BMP-2 was used, it was not possible to determine whether synergism occurred. When primary mouse calvarial osteoblasts were cultured under osteogenic conditions, Ro 13–6298 (1 to 100 nM) inhibited mineralization, but this was due primarily to an effect on cell morphology, rather than osteoblastic bone formation. In cultured neonatal, mouse calvarial bones, we have observed that 0.1 $\mu$M ATRA inhibits mRNA expression of the osteoblastic genes *Osteocalcin*, *Akp1*, *Runx2*, and *Procollagen $\alpha$(1) I* (89). This is in agreement with the study of Kneissel et al (75), which showed decreased periosteal mineralizing surfaces (independent of increased resorption), but an unaffected periosteal mineral apposition rate in rats treated with Ro 13–6298.

With regard to in vivo studies, we are aware of only 1 study where dynamic bone formation was assessed using double injections of alizarin and calcein (75). The authors found that treatment of 12-week-old rats with Ro 13–6298 (125 $\mu$g/kg) for 1 week substantially decreased mineralizing surfaces in periosteal areas but did not affect mineral apposition rate. This suggests that the number of bone-forming osteoblasts was reduced, but the activity of

**Table 5.** Summary of the Effects by Retinoids on Bone Resorption and Osteoclast Formation in Vivo

| Animal | Retinoid | Dose | Cortical Bone Mass | Trabecular Bone Mass | Subperiosteal Osteoclasts | Trabecular Osteoclasts | Ref. |
|---|---|---|---|---|---|---|---|
| Thyroparath. male rats | Ro 13–6298 | 25 $\mu$g sc 2–4 d | ↓ | ? | ↑ | ? | 166 |
| Male rats | Retinyl palmitate | 10 000 or 25 000 IU by stomach tube 21 d | ? | ? | ↑ | ? | 153 |
| Female rats | Ro 13–6298 | 125 $\mu$g/kg sc 4 d | ↓ | — | ↑ | ↓ | 75 |
| | ATRA | 120 mg/kg orally 4 d | ↓ | — | ? | ? | |
| Male rats | Retinyl palmitate + retinyl acetate | 1700 IU vitamin A/g pellet 8 d | ↓ | ↓ | ↑ | — | 76 |

Abbreviations: Thyroparath, thyroparathyroidectomized; ↓, decrease; ↑, increase; ?, not investigated; —, no effect.

the remaining osteoblasts was unaffected, a finding in agreement with in vitro observations suggesting that retinoids inhibit osteoblast differentiation. The authors ruled out the possibility that the decrease in mineralizing surfaces was a consequence of increased resorption by demonstrating that identical responses were seen when resorption was inhibited by alendronate. No effects of Ro 13–6298 on mineralizing surfaces or mineralizing apposition rate were seen in secondary or primary spongiosa of trabecular bone.

In rats with hypervitaminosis A caused by supplementation of their food with retinyl palmitate and retinyl acetate for 8 days, it has been reported that the bone formation markers in serum, procollagen type I N-terminal propeptide and osteocalcin, were decreased, indicating a general inhibitory effect by vitamin A on bone formation (76). However, increased immunohistochemical staining for osteocalcin was noted at the endosteal surfaces. This, together with increased mRNA expression of osteoblastic genes like *Akp1* and *Runx2* in bone marrow close to the endosteal surfaces, suggests locally increased osteoblastic activity. Supporting this view, the authors show formation of osteocyte-rich woven bone along the endosteal surfaces, an observation also made by Hough et al (153), but not by Kneissel et al (75). Bone formation has also been studied in 8-week-old mice fed a vitamin A-deficient diet after a hole was drilled in their femurs. Healing in this drill-hole model is due primarily to intramembranous bone formation. Reduced bone formation was observed (173). The authors also noted that vitamin A deficiency reduced the mRNA expression of *Bmp2*, *Collagen α1 type I*, and *Osteocalcin*. These data suggest that retinoids increase bone formation by enhancing the differentiation of osteoblasts. Similar results were obtained in another study analyzing bone formation in defects in the nonsuture-associated areas of parietal bones in adult mice (174). Mouse osteoblasts, adipose-derived stromal cells, and bone marrow-derived stromal cells were incubated ex vivo for 2–4 weeks with ATRA plus BMP-2 and then seeded in the top of the defects. In all cases, much more bone was formed after the addition of transplanted cells compared to when no cells were added; however, the study lacked transplanted control cells not incubated with ATRA and/or BMP-2, which would have helped assess the importance of these agents.

Zhang et al (171) used a model in which mouse embryonic fibroblasts were injected sc in nude mice. When these cells had been transfected with a BMP-9 vector, ectopic bone formation at the implantation site was found, a response that was significantly enhanced by cotransfecting the cells with either a *Rarα* or *Rxrα* vector; however, the trabecular bone formed was eventually resorbed by osteoclasts.

Shimono et al (175) have recently reported that retinoids inhibit heterotopic bone formation. Three experimental models were used in the study. In the first model, bone formation was assessed in a surgically created pouch in the calf muscle of 2-month-old mice after insertion of a collagen sponge containing BMP-2. This resulted in massive formation of heterotopic new bone. When these mice were treated with ATRA for 2 weeks, the amount of bone was reduced; when the animals were treated with the specific RAR-γ agonist, NRX204647, hardly any bone was formed. This suggests that treatment with retinoids could be used to reduce heterotopic bone formation resulting from invasive surgery or in seriously wounded soldiers.

In a second model, sc bone formation in young mice implanted with Matrigel containing BMP-2 was studied. It was observed that NRX204647 and 2 structurally different RARγ agonists, CD1530 and R667, were potent inhibitors of the heterotopic ossification. Treatment with ATRA or the RARα agonist, NRX195183, also inhibited the ossification, but these compounds were less effective and less potent. Lack of heterotopic ossification in RAR-γ-null mice was also observed, further pointing out the critical role of RARγ.

A similar type of bone formation associated with trauma or inflammation is seen in humans with the rare congenital disease fibrodysplasia ossificans progressiva (FOP). These patients exhibit an activation mutation in the BMP type I receptor, ALK2R206H. In the third model used by Shimono et al (175), heterotopic bone formation in the skin of transgenic mice with the human mutation was investigated. Bone formation was induced by injecting adeno-Cre im in a hind limb of mice carrying a Cre-inducible constitutively active ALK2 Q207D mutant. Treatment of these mice with CD1530 prevented the heterotopic bone formation at the site of adeno-Cre injections.

In the models employed by Shimono et al (175), new bone is formed by endochondral ossification, and it is not possible to determine exactly how the RARγ agonists act at the cellular level, but it is most likely that the compounds act on chondrogenesis rather than on osteoblastic differentiation or activity (176).

These studies should prompt future clinical studies in patients with disabling heterotopic bone formation, not only in those with FOP but also in patients with conditions predisposing for this type of pathological formation of bone, such as those operated on for total joint arthroplasties, traumatic brain injuries, paralysis, end-stage valvular heart disease, and in seriously wounded soldiers. Interestingly, isotretinoin (13-*cis* RA) treatment has been reported to decrease the incidence of heterotopic bone formation in FOP patients, although the use of this particular retinoid

caused a variety of side effects and was not considered clinically useful (177).

It appears that the roles retinoids play in osteoblast differentiation and activity in different parts of the skeleton are still elusive, and more studies are needed to assess how these compounds affect the anabolic side of bone remodeling.

## XII. Conclusions

Supplementation of the diet with vitamins is a common occurrence, and there is debate over whether increased vitamin A intake might promote skeletal fragility. Some studies have suggested that increased vitamin A intake may decrease BMD and promote hip fracture; however, other studies have not shown increased bone loss or increased fracture risk, and in some instances, protection from bone loss by vitamin A has been suggested. Vitamin D plays a major role in calcium absorption and mineral homeostasis. Vitamin D deficiency is common, and some studies have suggested that the risk of osteoporosis and fracture may increase when increased vitamin A intake occurs in individuals with low vitamin D levels. It is possible that an increased risk of osteoporosis and fracture might exist for increased vitamin A intake and/or increased intake in the face of low vitamin D, but it appears that additional in vivo animal studies and studies in humans to confirm or dispel these possibilities will be necessary before clearer estimates of risk emerge.

Increased osteoclastic resorption of periosteal bone is a well-documented in vitro consequence of excess vitamin A. Calvarial bone is considered to be a good model of periosteal resorption of cortical bone, and it has been established that ATRA is a good in vitro stimulator of RANKL, osteoclastogenesis, and resorption in cultured calvarial bones. In contrast, there is also ample evidence that ATRA is a potent in vitro inhibitor of osteoclastogenesis in cell culture systems employing progenitors from either mouse bone marrow or spleen, or human peripheral blood. Periosteal resorption of cortical bone has also been firmly established as an in vivo consequence of excess vitamin A in experimental animals, as well as in humans. Furthermore, available evidence suggests that excess vitamin A also decreases endosteal blood flow, reduces endosteal osteoclast formation, and promotes endosteal mineralization (Figure 5A).

Effects of vitamin A on cancellous bone resorption are less clear. Of the 3 animal studies investigating cancellous bone, 1 has shown a decrease in trabecular osteoclasts with no change in trabecular bone mass (Figure 5B) (75). These data appear to be in agreement with in vitro investigations indicating inhibition of osteoclast progenitors from bone marrow; however, the 2 other studies have reported decreases in trabecular bone mass (75, 147) and, in 1 of these investigations, no change in trabecular osteoclasts. Thus, it appears that additional in vivo animal studies will be necessary before these different observations can be clarified.

Most of the studies on effects of vitamin A on osteoblast differentiation and formation indicate that vitamin A promotes osteoblast differentiation and bone formation (Figure 5C). Interestingly, injury-induced heterotopic bone formation can be inhibited by RAR$\gamma$ signaling by retinoids.

In postmenopausal osteoporosis, the prevailing idea has been that loss of trabecular bone was the main cause of increased fracture, but evidence is now accumulating to suggest that loss of cortical bone might be the major determinant of fracture risk in these patients (178). Cortical bone thinning occurring in humans as a consequence of increased vitamin A intake would also cause bone fragility, but unlike osteoporosis in the postmenopausal female, where cancellous bone loss is significant, cancellous bone loss may not play as prominent a role in vitamin A excess. In the past, DXA has been employed to measure BMD and has proven to be valuable clinically, especially in the postmenopausal female, but DXA does not distinguish between cortical and trabecular bone. To what extent past use of DXA for BMD measurements using criteria normally employed for conditions like postmenopausal osteoporosis may have led to differing outcomes in some of the studies evaluating vitamin A intake, BMD, and fracture risk is unclear, but the use of more specific tools to distinguish between trabecular and cortical bone appears to be essential.

Other than for the suggestion of low levels of vitamin D, it is not known presently whether there might be other specific conditions or dietary and environmental differences that might enhance bone loss in the presence of increased concentrations of vitamin A. Valuable insight for preventing future harmful effects of vitamin A on bone might be gained by additional correlative studies of vitamin A intake. Animal studies using state-of-the-art techniques to access site-specific effects of vitamin A on bone resorption and formation, BMD, and bone fragility also seem clearly warranted, as do studies in mice with cell-specific deletions of different RARs and RXRs. Genetic studies evaluating the relationships of RAR and RXR genes to bone mass and fragility fracture in the human should additionally be pursued. Moreover, prospective studies in humans using peripheral quantitative computed tomography for evaluation of specific effects in cortical and trabecular bone appear to be essential if we are to gain

**Figure 5.**

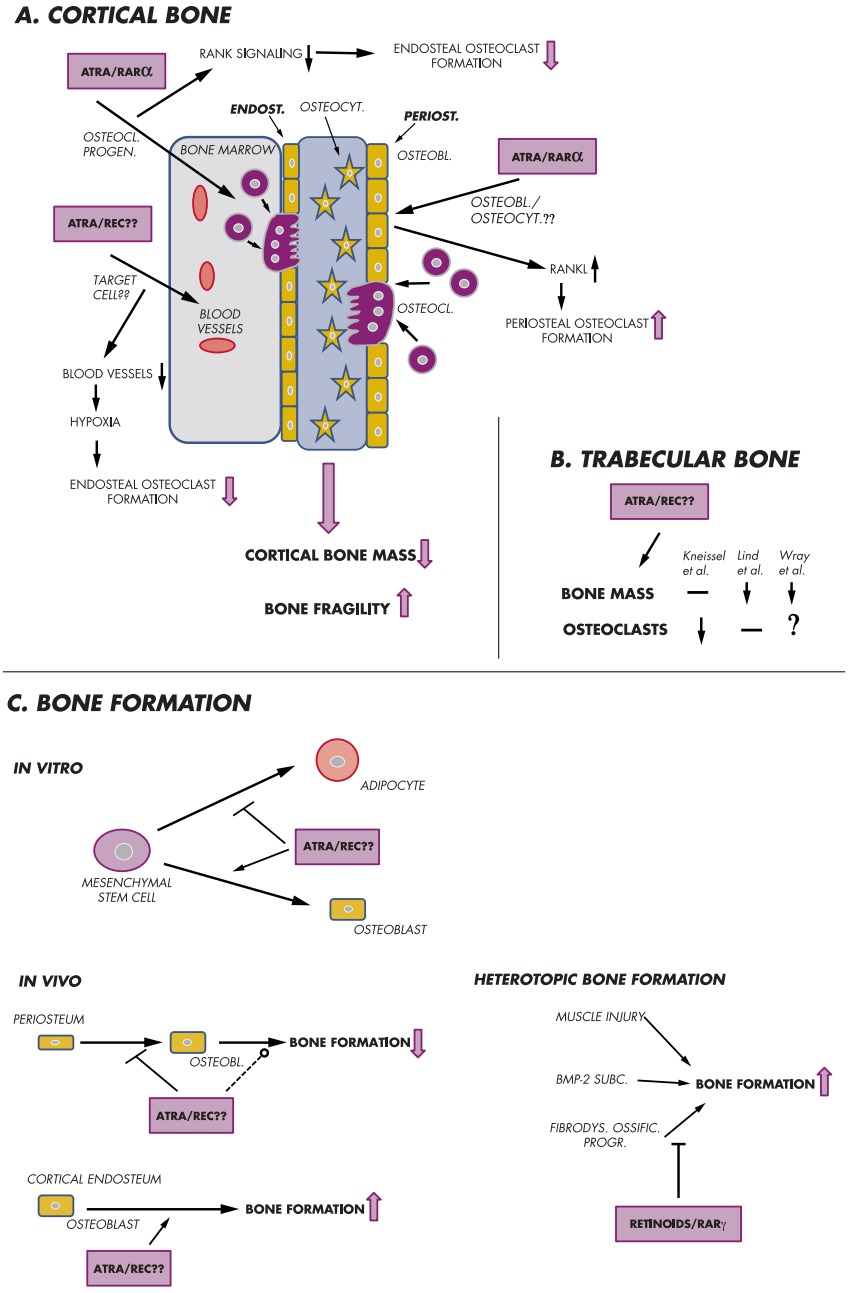


Figure 5. Hypervitaminosis A causes decreased cortical bone mass, whereas effects on trabecular bone still remain elusive. Changes in cortical bone are associated with effects on osteoclast formation as well as on bone formation. A, Hypervitaminosis A increases the number of osteoclasts on periosteal surfaces. This effect is due to an increased RANKL/OPG ratio mediated by RARα, most likely in osteoblasts or osteocytes. At the endosteal surface of cortical bone, it seems as if vitamin A decreases osteoclasts, which may be due to decreased numbers of microvessels in bone marrow due to hypoxia. Another explanation is that differentiation of osteoclast progenitors in bone marrow is inhibited because of RARα-mediated inhibition of RANK signaling. B, Effects by vitamin A on trabecular bone are less well studied. Two studies suggest that vitamin A has no effect on trabecular bone mass, whereas 1 study did not observe a change in bone mass. Differences were also noted in the number of osteoclasts. C, Data obtained in vitro suggest that vitamin A inhibits adipocyte differentiation and stimulates osteoblast differentiation, with vitamin A acting synergistically with BMP-2 in both instances. One in vivo study using dynamic histomorphometry indicates that vitamin A decreases the number of bone-forming osteoblasts on periosteal surfaces with no effect on the activity of the remaining osteoblasts, which suggests that vitamin A has an inhibitory effect on periosteal bone formation. Another morphological study indicates that vitamin A stimulates formation of atypical woven bone at the endosteal surfaces. Interestingly, retinoids have been shown to inhibit heterotopic bone formation in 3 different studies.

a better understanding of the risk that increased vitamin A intake might pose to skeletal health.

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