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Review

Bone effects of vitamin D – Discrepancies between *in vivo* and *in vitro* studies

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ABSTRACT

Vitamin D was discovered as an anti-rachitic agent, but even at present, there is no direct evidence to support the concept that vitamin D directly stimulates osteoblastic bone formation and mineralization. It appears to be paradoxical, but vitamin D functions in the process of osteoclastic bone resorption. In 1952, Carlsson reported that administration of vitamin D_3 to rats fed a vitamin D-deficient, low calcium diet raised serum calcium levels. Since the diet did not contain appreciable amounts of calcium, the rise in serum calcium was considered to be derived from bone. Since then, this assay has been used as a standard bioassay for vitamin D compounds. Osteoclasts, the cells responsible for bone resorption, develop from hematopoietic cells of the monocyte-macrophage lineage. Several lines of evidence have shown that the active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$] is one of the most potent inducers of receptor activator of NF-κB ligand (RANKL), a key molecule for osteoclastogenesis, *in vitro*. In fact, $1\alpha,25(OH)_2D_3$ strongly induced osteoclast formation and bone resorption *in vitro*. Nevertheless, $1\alpha,25(OH)_2D_3$ and its prodrug, Alfacalcidol (1α-hydroxyvitamin D_3) have been used as therapeutic agents for osteoporosis since 1983, because they increase bone mineral density and reduce the incidence of bone fracture *in vivo*. Furthermore, a new vitamin D analog, Eldecalcitol [2β-(3-hydroxypropoxy)-1α,25$(OH)_2D_3$], has been approved as a new drug for osteoporosis in Japan in January 2011. Interestingly, these beneficial effects of *in vivo* administration of vitamin D compounds are caused by the suppression of osteoclastic bone resorption. The present review article describes the mechanism of the discrepancy of vitamin D compounds in osteoclastic bone resorption between *in vivo* and *in vitro*.

1. Introduction

Bone remodeling is a dynamic process orchestrated by bone-forming osteoblasts and bone-resorbing osteoclasts. In normal bone remodeling, osteoclastic bone resorption is followed by osteoblastic bone formation through a coupling mechanism [1]. Osteoporosis is a common skeletal disease involving a decrease in bone mineral density (BMD),[1] bone quality, and bone strength [2,3]. Osteoporosis is caused by an imbalance of bone resorption and bone formation, with the former exceeding the latter.

It is well recognized that, in higher vertebrates including humans, serum calcium levels are tightly regulated and maintained at 9–10 mg/dL [4]. Intestine, bone and kidney are the three major organs involved in this calcium homeostasis [4]. Vitamin D plays a major role in regulating serum calcium homeostasis in concert with parathyroid hormone (PTH). Of great importance is the fact that PTH is required for calcium mobilization from bone and for renal reabsorption of calcium, but is not directly required for intestinal calcium transport system [4] (Fig. 1). The total amount of calcium present in adult humans is estimated to be approximately 1000 g/body, and 99% of them are stored in bone. Bone calcium is mobilized by osteoclasts into blood stream from calcified bone. Thus, it is considered that bone is a storehouse of calcium in the body [4].

2. Role of vitamin D in bone formation and mineralization

Vitamin D was originally discovered as an anti-rachitic agent capable of preventing a failure of bone mineralization [5]. A deficiency of vitamin D results in rickets in the young and osteomalacia in the adults [5]. Administration of vitamin D to rachitic animals and

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[1] *Abbreviations used:* BMD, bone mineral density; PTH, parathyroid hormone; $1\alpha,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; FGF23, fibroblast growth factor 23; VDR, vitamin D receptor; IL-6, interleukin 6; PGE2, prostaglandin E2; OSM, oncostatin M; LIF, leukemia inhibitory factor; ODF, osteoclast differentiation factor; M-CSF, macrophage colony-stimulating factor; TNF, tumor necrosis factor; c-Fms, M-CSF receptors; OPG, osteoprotegerin; CKD, chronic kidney disease; OVX, ovariectomized; NTX, N-telopeptide of type 1 collagen; BAP, bone alkaline phosphatase; QOPs, quiescent osteoclast precursors; RANKL, receptor activator of NF-κB ligand; RANKLS, RANKL-positive cell surface.

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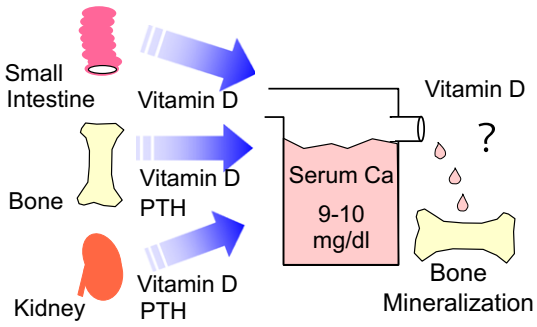

Fig. 1. A diagrammatic representation of the classical actions of vitamin D to maintain serum calcium homeostasis. While vitamin D is the sole substance to stimulate intestinal absorption of calcium, vitamin D and PTH working in concert are necessary to mobilize calcium from bone and conserve calcium from urine. It was postulated that vitamin D directly stimulates osteoblastic bone formation and mineralization, but even now there is no direct evidence of this.

humans cures impaired bone mineralization. It was therefore postulated that vitamin D directly stimulates osteoblastic bone formation and mineralization, but even now there is no direct evidence of this. In fact, early work by Rowland and Kramer [6], later work by Lamm and Neuman [7], and more recent work by Underwood and DeLuca [8] clearly demonstrated that vitamin D does not play a significant role in the actual mineralization process of the skeleton.

Vitamin D_3 is first metabolized to 25-hydroxyvitamin D_3 [25(OH)D_3] in the liver, then in the kidney to 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] [9]. In this pathway, the renal 1α-hydroxylation reaction is a rate-limiting step in the production of 1α,25$(OH)_2D_3$ [9] and it is strictly regulated by PTH [10], fibroblast growth factor 23 (FGF23) [11], and the vitamin D status of animals [12]. 1α,25$(OH)_2D_3$ is now recognized as a steroid hormone that plays a critical role in maintaining calcium homeostasis through the nuclear vitamin D receptor (VDR) [4].

Yoshizawa et al. [13] generated VDR knockout mice, and found no appreciable defects during development before weaning. After weaning, however, the VDR knockout mice failed to thrive, and hypocalcemia and infertility resulted. Both bone formation and mineralization were severely impaired as a typical feature of vitamin D-deficient type II rickets. Most of the animals died within 25 weeks after birth due to severe hypocalcemia. Unexpectedly, when the VDR knockout mice were fed a high-calcium diet, they developed normally even at week 50. Bone formation and mineralization in the VDR knockout mice fed a high calcium diet were completely reestablished, though severe alopecia remained. Furthermore, Tanaka and Seino [14] examined direct action of vitamin D on bone formation and mineralization by transplanting bone isolated from VDR knockout mice into wild type mice. The VDR-deficient bone transplanted to the wild type mice showed excessive bone formation and mineralization in normocalcemic conditions, suggesting that vitamin D rather negatively regulates bone formation and mineralization. From these results, it was concluded that the stimulating effect of 1α,25$(OH)_2D_3$ on bone formation and mineralization is rather indirect, occurring through stimulation of the intestinal absorption of calcium [13].

3. Discovery of bone mobilizing effect of vitamin D

Although it appears paradoxical, vitamin D induces bone resorption. Carlsson [15] and Bauer et al. [16] were the first to realize that a major function of vitamin D is to induce the mobilization of calcium from bone, making calcium available to the extracellular fluid upon demand by the calcium homeostatic system. Carlsson [15] showed that, when hypocalcemic rats maintained for 3 weeks on a vitamin D-deficient, low calcium diet were orally given 100 IU (2,5 μg) of vitamin D_3, their serum calcium levels were increased from 5 to 8 mg/dL three days after its administration. Parathyroidectomy two hours prior to vitamin D_3 administration abolished the increase in serum calcium levels. Since the diet did not contain any appreciable amounts of calcium, he concluded that vitamin D stimulates mineral mobilization from calcified bone in concert with PTH. Since then, this assay method has been used as a standard bioassay for vitamin D compounds [17].

The metabolite of vitamin D_3 responsible for bone mineral mobilization was 1α,25$(OH)_2D_3$. Using an *in vitro* organ culture system, Raisz et al. [18] reported that both 1α,25$(OH)_2D_3$ and 25(OH)D_3 increased the release of ^{45}Ca from prelabeled bone into culture medium *in vitro*, but 1α,25$(OH)_2D_3$ was 80 times more potent than 25(OH)D_3. From these results, it is concluded that the metabolite of vitamin D_3 which stimulates bone mineral mobilization is indeed 1α,25$(OH)_2D_3$ [19].

4. Discovery of the RANKL–RANK–OPG system

In 1981, Rodan and Martin [20] proposed that osteoblasts or bone marrow stromal cells may intervene in the process of osteoclastic bone resorption. Their argument such a mechanism was based on the observations that first, bone-resorbing hormones and cytokines have their receptors in osteoblastic cells but not in osteoclasts, and second, the relative binding potencies of these bone-resorbing factors to their respective receptors in osteoblasts resemble those in inducing bone resorption. Based on the concept proposed by Rodan and Martin, Takahashi et al. [21] established an efficient mouse co-culture system of primary osteoblasts isolated from calvaria and spleen cells to recruit osteoclasts. A number of multinucleated osteoclasts were formed in response to 1α,25$(OH)_2D_3$ in this co-culture system. Cell-to-cell contact between spleen cells and osteoblastic cells appeared important for both osteoclast formation and activation [21].

In 1992, we proposed a working hypothesis for the mechanism of osteoclastogenesis based on the extensive studies using the co-culture system [22] (Fig. 2). Various bone-resorbing factors including 1α,25$(OH)_2D_3$, PTH and interleukin 6 (IL-6) together with soluble IL-6 receptor (IL-6 + sIL-6R) appeared to act commonly on osteoblastic cells, but not hematopoietic osteoclast precursors in the co-cultures. These bone-resorbing factors were classified into three categories in terms of their signal transduction pathways: VDR-mediated signals [1α,25$(OH)_2D_3$, protein kinase A-mediated signals [PTH, prostaglandin E_2 (PGE_2)], and gp130-mediated signals [IL-6, IL-11 oncostatin M (OSM), and leukemia inhibitory factor (LIF)]. We proposed that a membrane-bound factor named osteoclast differentiation factor (ODF) which is commonly induced on the plasma membrane of osteoblastic cells in response to these bone-resorbing factors, mediates an essential signal to osteoclast progenitors for their differentiation into mature osteoclasts [22]. Chambers et al. [23] also named a similar factor "SOFA" (stromal cell-derived osteoclast forming activity) and performed its search (Fig. 2). Osteoclast progenitors having ODF receptor recognize ODF by cell-to-cell contact and differentiate into osteoclasts. Macrophage colony-stimulating factor (M-CSF) produced by osteoblastic cells is also indispensable for both proliferation and differentiation of osteoclast progenitors [24,25]. Thus, osteoblastic cells are important for osteoclastogenesis in two different ways: one is the production of M-CSF, and the other is the induction of a membrane-bound factor ODF [22]. Production of the former occurs even in the absence of bone-resorbing factors, but the induction of ODF strictly depends on the presence of those factors [22].

In 1998, we finally identified the structure of ODF in collaboration with Yasuda and Higashio of the Snow Brand Milk Products in Japan [26]. By screening a cDNA expression library of ST2 cells, we cloned