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Vitamin D3, vitamin K2, and warfarin regulate bone metabolism in human paranasal sinus bones

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SUMMARY

Several recent studies have indicated that the paranasal sinus bones undergo pathophysiological changes in patients with chronic sinusitis. We examined the mineralization activity of osteoblasts and the production of osteocalcin and cytokines in cultured human osteoblasts derived from ethmoidal bones treated with vitamin D3, vitamin K2, and warfarin to investigate the metabolic effects of these treatments on paranasal sinus bones. In the bones treated with vitamin D3 plus vitamin K2, osteocalcin production and the ratio of the mineralization of osteoblasts were increased. Warfarin inhibited the promotive effects of vitamin K2 in the presence of vitamin D3. With regard to TGF-β production, there was quite a difference in response depending on the isoforms. In conclusion, we have demonstrated that these vitamins and warfarin may be useful in improving bone metabolism in paranasal sinus bones, and may additionally improve the pathogenesis of chronic sinusitis.

Key words: osteoblast, vitamin D3, vitamin K2, warfarin, TGF-β

INTRODUCTION

Pathophysiological changes in chronic sinusitis have previously been studied primarily from the perspective of mucous membranes. However, it has recently been suggested that not only sinus mucosa but also sinus bones may play an important role as catalysts of chronic sinusitis. Kennedy et al. [1] have reported that individuals undergoing surgery for chronic sinusitis show evidence of marked acceleration in paranasal sinus bone physiology, with histological changes including new bone formation, fibrosis, and the presence of inflammatory cells. Khalid et al. [2] have reported that in the paranasal sinus bones of rabbits, the inflammation spreads through the widened Haversian canal system within the bone. However there have been few reports of cytokine production from osteoblasts derived from sinus bones.

In contrast, there have been numerous studies on the relation between bone metabolism and the production of cytokines such as IL-1β, IL-6, TGF-β, TNF-α, and BMP in osteoblasts from other organs [3-6]. In addition, reports on other organs have also shown that vitamin D3 and vitamin K2 stimulation promote bone metabolism [7-9]. Vitamin D3 plays a role in the regulation of the early stages of human osteoblast differentiation [10]. Vitamin K2 may have important positive effects on the development and maintenance of bone through its role in promoting carboxylation of the matrix protein, osteocalcin [11,12]. Therefore, these vitamins are clinically used to improve bone metabolism.

However, the previous results were achieved using rodent bones and human femurs [13-15], and there has been no study using vitamin D3 and K2 stimulations in order to improve the metabolism of human paranasal sinus bones. Osteoblasts contribute to marked acceleration of paranasal sinus-bone physiology under chronic infection, but the mechanism of occasional sinus-bone remodeling, such as bone thickness and bone erosion, remains vague, so we hypothesized that vitamin D3, vitamin K2 and warfarin (a vitamin K antagonist) stimulation in osteoblasts from the sinus may affect the metabolism of paranasal sinus bones and cytokine production from sinus-bone osteoblasts. Therefore, in the present study we investigated the mineralization activity of osteoblasts and the production of cytokines in a culture of osteoblasts obtained from the paranasal sinus bones of patients with chronic sinusitis in order to elucidate the metabolic effects of vitamin D3 and K2 and warfarin on these osteoblasts.

MATERIALS AND METHODS

Cell culture

For the purposes of this study, we followed the explant-outgrowth cell culture method described by Ishino et al. [16]. Ethmoidal sinus bones were obtained from 6 subjects (4 males, 2 females, 29-72 years of age, 3 smokers, 3 non-smokers) with chronic sinusitis at the time of sinus surgery. Informed consent was obtained from all individuals included in this study. Bones were washed extensively in sterile phosphate buffered saline (PBS, pH 7.2) to remove blood and debris. The explants were then dissected into 1-3 mm^2 pieces and placed in 35-mm dishes containing Dulbecco's modified Eagle's medium supplement-

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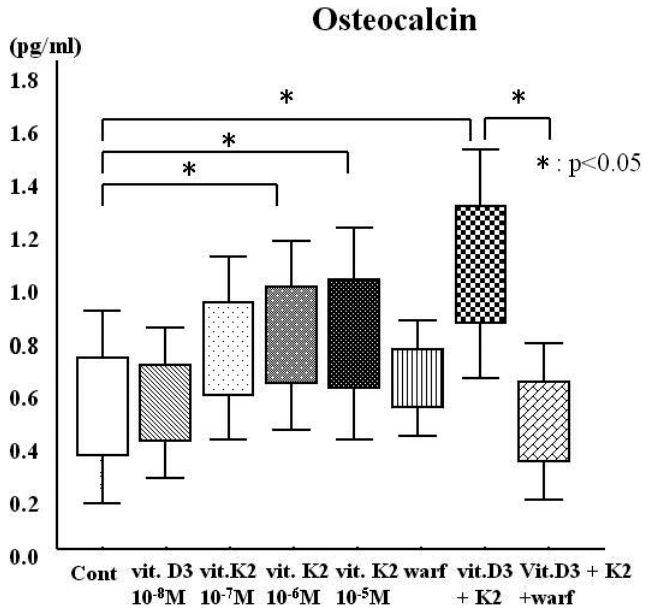

Figure 1. Concentrations of osteocalcin in the media were measured on the 20th day after stimulation. Concentrations of osteocalcin in the high-dose vitamin K2 (10^{-6}M, 10^{-5}M) groups and the vitamin D3 (10^{-8}M) plus vitamin K2 (10^{-6}M) group were significantly higher than that in the control group. The concentration of osteocalcin in the vitamin D3 plus vitamin K2 plus warfarin group was significantly lower than that in the vitamin D3 plus vitamin K2 group.

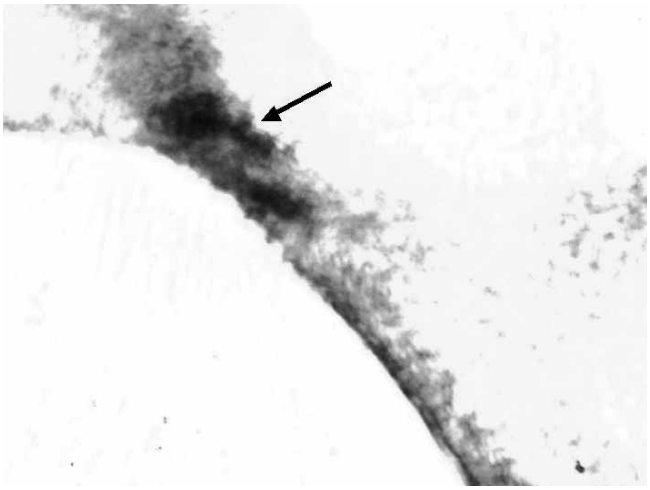

Figure 2. Osteoblast cell layers were stained for mineralization by the von Kossa method.

ed with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), 200 U/ml penicillin, and 200 mg/ml streptomycin (Meiji Seika, Tokyo, Japan), and 5 mg/ml fungizone (Bristol-Myers Squibb, Tokyo, Japan). The explants were incubated at 37°C in 95% air and 5% CO2, and the culture medium was changed every 3-4 days. The osteoblasts in the explant culture were harvested with trypsine-EDTA over 5-10 min at 37°C when they had reached confluence, and were seeded in a 25 cm^2 flask. The osteoblasts at the second passages were also harvested upon reaching confluence and were seeded in 24-well plates at 104 cells/well.

Treatments with vitamin D3, vitamin K2, and warfarin

Each well was filled with one of eight types of medium: normal medium, medium including vitamin D3 (10^{-8}M), medium including vitamin K2 (10^{-5}M: high dose, 10^{-6}M: medium dose, 10^{-7}M: low dose), medium including vitamin D3 (10^{-8}M) and vitamin K2 (10^{-6}M), medium including warfarin ($2.5x10^{-5}$M), and medium including vitamin D3 (10^{-8}M) vitamin K2 (10^{-6}M) and warfarin ($2.5x10^{-5}$M). These physiological concentrations were slightly modified from previously reported methods [8,17]. Vitamin D3 was purchased from Wako (Osaka, Japan) and vitamin K2 and warfarin were kindly supplied by Eisai Co. (Tokyo, Japan).

Mineralization activity assay

At 20 days after treatment, the mineralization activities were examined in two ways. The osteocalcin concentrations were measured using enzyme-linked immunoassay (ELISA). The ratio of the mineralization area after von Kossa staining was analyzed with IP lab software after visualization with an optic microscope.

Cytokine assay

Cytokines were measured using an enzyme-linked immunoassay (ELISA) kit (R&D systems, Minneapolis, MN, USA) at 20 days after treatment. To measure the production of cytokines (TGF-β1, TGF-β2, and IL-6) in culture medium, medium was exchanged for serum-free medium 24 hours before the collection time.

Statistics

Statistical analyses were performed using the Wilcoxon signed rank test.

RESULTS

Concentrations of osteocalcin in the treatment groups were compared to those in the control. The concentrations of osteocalcin in the high-dose vitamin K2 (10^{-6}M, 10^{-5}M) groups and vitamin D3 (10^{-8}M) plus vitamin K2 (10^{-6}M) group were higher than those in the control. The concentration of osteocalcin in the vitamin D3 plus vitamin K2 plus warfarin group was significantly lower than that in the vitamin D3 plus vitamin K2 group (Figure 1). The ratio of the mineralization area after von Kossa staining (Figure 2) was also compared to that of the control. The ratios of the mineralization area in the vitamin K2 (10^{-5}M) group and the vitamin D3 plus vitamin K2 group were significantly higher than those in the control. The ratio of the mineralization area in the vitamin D3 plus vitamin K2 plus warfarin group was significantly lower than that in the vitamin D3 plus vitamin K2 group (Figure 3).

Figure 4 shows the TGF-β1 production of cultured osteoblasts in different media. Concentrations of TGF-β1 in the vitamin

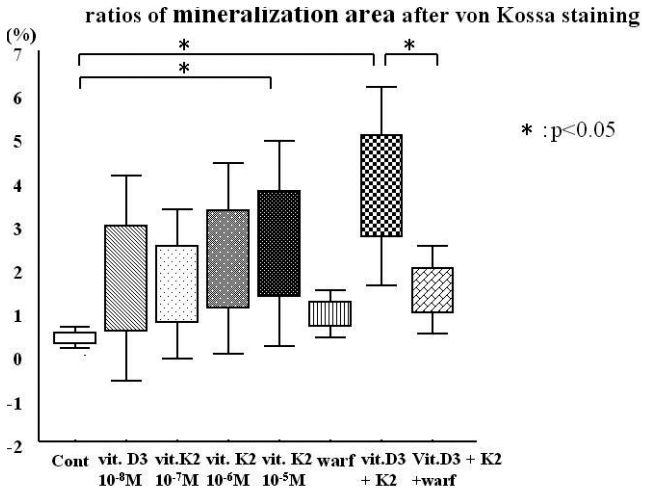

Figure 3. The ratios of the mineralization area after von Kossa staining were measured on the 20th day after stimulation. The ratios of the mineralization area in the vitamin K2 (10^{-5}M) group and the vitamin D3 plus K2 group were significantly higher than that in the control group. The ratio of the mineralization area in the vitamin D3 plus vitamin K2 and warfarin group was significantly lower than that in the vitamin D3 plus vitamin K2 group.

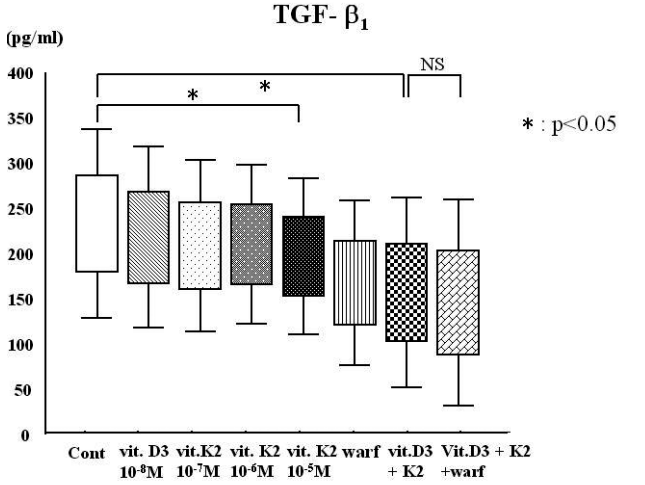

Figure 4. TGF-β1 production in the media was measured on the 20th day after stimulation. TGF-β1 productions in the vitamin K2 (10^{-5}M) group and the vitamin D3 plus vitamin K2 group were significantly lower than that in the control group.

K2 (10^{-5}M) group and the vitamin D3 plus vitamin K2 group were significantly lower than that in the control. The TGF-β1 concentration in the warfarin group was not significantly different from that in the control. For TGF-β2, the concentration in the vitamin D3 plus vitamin K2 group was significantly higher than that in the control. In all other groups the concentrations of TGF-β2 were not significantly different from that in the control (Figure 5). The concentrations of TGF-β1 were three times higher than those of TGF-β2 in all groups. In the analysis of the concentrations of IL-6, there were no significant differences among the groups (data not shown).

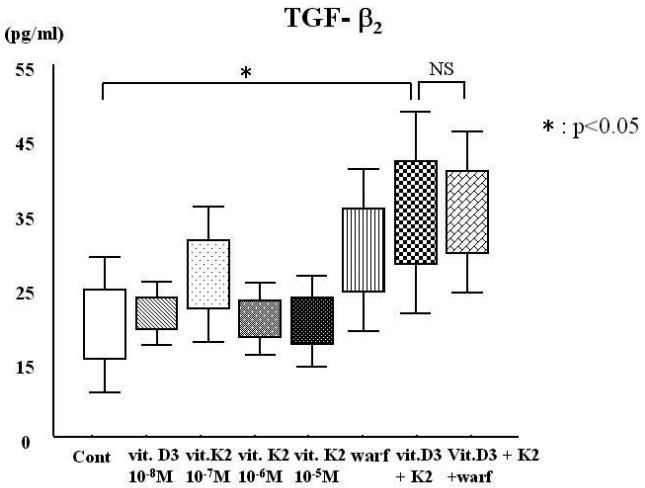

Figure 5. TGF-β2 production in the media was measured on the 20th day after stimulation. TGF-β2 production in the vitamin D3 and vitamin K2 group was significantly higher than that in the control group.

DISCUSSION

Bone tissue includes three types of cells (osteocytes, osteoblasts, osteoclasts). Bone remodeling keeps the three types in balance. But when bone remodeling is disordered, vitamin D3 and vitamin K2 can generally be used to promote bone metabolism. Of these vitamins, it has become well known that vitamin D3 plays a role in the regulation of the early stages of human osteoblast differentiation [10], and that vitamin K2 promotes bone formation and inhibits bone resorption. Previous studies have demonstrated that vitamin K2 plays an important positive role in the development and maintenance of bone through its role in promoting carboxylation of osteocalcin, whose matrix protein is a marker of bone formation and has an affinity for hydroxyapatite [11,18-20]. Some authors have demonstrated that the use of vitamin D3 and vitamin K2 together promotes mineralization in human periosteal osteoblasts from the ulna [9,17]. In addition, one of these studies demonstrated that warfarin, which is an antagonist of vitamin K, strongly inhibits epoxide reductase in the vitamin K cycle in osteoblasts [9].

We have found in the present study that the amount of osteocalcin released by osteoblasts increased after stimulation with high doses of vitamin K2 and vitamin D3 plus vitamin K2, and that the ratios of mineralization of osteoblasts increased after stimulation with a high dose of vitamin K2 and vitamin D3 plus vitamin K2. These results indicate that stimulation with vitamin D3 and vitamin K2 together affects osteoblasts in the paranasal sinus, much as it has previously been shown to affect diaphysial bones; they also suggest that vitamin K2 in a high dose or in the presence of vitamin D3 promotes the mineralization of osteoblasts, while vitamin D3 alone does not. Choi et al. [21] has reported that the active expression of the osteocalcin gene for the late period (day16-28) correlates with the formation of bone nodules, and that the expression patterns of

the inducer of the mineralization (osteocalcin) of bone cells are mutually exclusive. In our data, the patterns of Figure 1 and Figure 3 were similar, so it was presumed that there is a correlation between the amount of osteocalcin released by osteoblasts and the mineralization of osteoblasts, as reported previously, and that the mineralization of osteoblasts in paranasal sinus bones is promoted as a result of the increase in osteocalcin after stimulation with these vitamins.
We have also shown that warfarin inhibits the promotive effects of vitamin K2 in the presence of vitamin D3 on the mineralization of osteoblasts. It was presumed that warfarin inhibits the effects of vitamin K2 on osteoblasts in paranasal sinus bones as much as in diaphysial bones.

To elucidate the metabolism of paranasal sinus bones in the context of stimulation by these vitamins and warfarin, we also investigated cytokine levels of TGF-β1, 2, and IL-6 in osteoblast cultures. In bone metabolism studies up to the present, the production and expression of cytokines such as IL-1β, IL-6, TGF-β, TNF-α, BMP etc. have been well investigated only in osteoblasts cultured from other organs [3-6].
The bone matrix is one of the largest storages of TGF-β [22]. TGF-β is an important cytokine secreted by bone cells, eosinophils and so on, and it affects the growth, development, and proliferation and differentiation of bone cells [23] and fibroblasts [24].
TGF-β has three isoforms, TGF-β1, TGF-β2, and TGF-β3. Miller has reported that the biological activities of these isoforms are almost the same [25]. In a study using primary bone cells cultured from the iliac crest, Bismar et al. [26] reported that the predominant TGF-β isoform secreted by human bone cells in culture is TGF-β1. In previous studies of the effects of TGF-β1 function on osteoblasts, TGF-β1 has been shown to enhance mineralization of human osteoblasts and to increase osteoblastic cell proliferation [13,27]. In contrast, Joyce et al. [28] has reported that TGF-β2 plays a more active role in osteogenesis and chondrogenesis in vitro than TGF-β1. Horner et al. [29] has reported that TGF-β2 is the most frequently detected isoform at sites of bone formation.
Additionally, IL-6 is an important cytokine that participates in bone resorption [30-32]. In reports about vitamin D3 and IL-6 production, controversial opinions have been asserted. Tran et al. [33] has reported that vitamin D3 stimulates IL-6 production while Kozawa et al. [34] has reported that vitamin D3 inhibits IL-6 synthesis. There has also been a report that vitamin D3 has only weak effects on IL-6 production [35].

In this study, we showed that concentrations of TGF-β1 in the high-dose vitamin K2 group and the vitamin D3 plus vitamin K2 group were significantly lower than that in the control, and that the concentration of TGF-β2 in the vitamin D3 plus vitamin K2 group was significantly higher than that in the control. Additionally, the concentrations of TGF-β1 were found to be three times higher than those of TGF-β2 in all groups, and warfarin had no observable effect on TGF-β1 and 2 in osteoblasts. Our data also revealed no significant change in IL-6 production following vitamin and warfarin stimulation. From these data, with regard to relations between TGF-β production and osteogenesis parameters (osteocalcin, mineralization ratio) in osteoblasts of paranasal sinus bones under vitamin and warfarin stimulation, we can deduce that TGF-β2 is the primary isoform at sites of bone formation compared to TGF-β1 under vitamin stimulation, despite the concentrations of TGF-β2 being low, and that there is no relation between TGF-β production and osteogenesis parameters under warfarin stimulation. In addition we can deduce there is no relation between IL-6 production and osteogenesis parameters under vitamin and warfarin stimulation, even though other studies have offered conflicting results.

The results of this study indicate that vitamin K2 in the presence of vitamin D3 may be a useful substrate to promote bone metabolism in the paranasal sinuses. It has been recognized that vitamins have low toxicity. Therefore, these results suggest that vitamin D3 and vitamin K2 are good substrates as osteogenesis inducers, and may be clinically useful in treating patients after surgery for bony-wall-fracture-related sickness such as the fracture of facial bones, nasal bones, the nasal septum, and so on.
Additionally, in nasal polyp formation, a previous report showed that TGF-β1 induces fibroblast- proliferation and differentiates fibroblasts to myofibroblasts [24]. Myofibroblasts are the main cells to producing the extra-cellular matrix in nasal polyps. Lee et al. reported that TGF-β1 may contribute to eosinophil and mast cell migrations into nasal polyp tissue [36]. TGF-β2 has the same potential in terms of its biological effects [25], but this study showed that the level of production of TGF-β1 is higher than that of TGF-β2. Down regulation of TGF-β1 production in osteoblasts from vitamin K2 plus vitamin D3 stimulation suggests that these vitamins may become growth inhibitors of nasal polyps in chronic sinusitis.

With regard to warfarin, warfarin inhibits the promotive effects of the mineralization of osteoblasts. In chronic sinusitis, inflammation induces the mineralization of osteoblasts. The results of this study indicate that warfarin may be a useful substrate to inhibit bone metabolism in the paranasal sinuses. Though it has been noted that warfarin promotes bleeding, these results suggest that warfarin is a good substrate as an osteogenesis inhibitor, and may be clinically useful to prevent bony stenosis of the ostium after operations for chronic sinusitis.

In conclusion, we have demonstrated that the combination of vitamin D3 plus vitamin K2 promotes the mineralization of osteoblasts cultured from the paranasal sinus bone. In addition, we have demonstrated that warfarin inhibits the effects of vitamin K2 in the presence of vitamin D3 on cultured

paranasal sinus osteoblasts. With respect to TGF-β production, vitamin D3 plus vitamin K2 stimulation on osteoblasts produces opposite effects according to differences in the isoforms. Further research may elucidate both the mechanism of the metabolism of paranasal sinus bones and the pathogenic role of osteoblasts in chronic sinusitis and may demonstrate the availability of these vitamins and warfarin to improve bone metabolism in the field of the paranasal sinus.

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