Vitamin D decreases the secretion of eotaxin and RANTES in nasal polyp fibroblasts derived from Taiwanese patients with chronic rhinosinusitis with nasal polyps

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Abstract  Eosinophils are important inflammatory cells involved in the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP). Vitamin D and its derivatives, in addition to their classic role as regulators of electrolytes homeostasis, have modulatory effects in immunological and inflammatory responses. Such properties suggest that vitamin D might also play a role in inflammatory airway diseases such as CRSwNP. In this study, we investigated the effect of vitamin D derivatives (calcitriol and tacalcitol) on the secretion of eotaxin and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), the two major eosinophil chemoattractants, in fibroblasts derived from the polyps of Taiwanese CRSwNP patients. Patients diagnosed with eosinophilic CRSwNP but without malignancies or asthma and undergoing elective endoscopic sinus surgery were recruited. Three primary fibroblast cultures were established using the polyp specimens obtained from these patients. The third to eighth passages of the fibroblasts were used for in vitro studies. Nasal polyp-derived fibroblasts were stimulated with IL-1\textbeta (10 ng/mL) for 24 hours, followed by replacement with media alone or with calcitriol or tacalcitol (10\textmu M) and incubation for another 24 hours. After the treatments, the

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levels of secreted eotaxin and RANTES were evaluated by ELISA assays. The results showed that IL-1β could substantially stimulate the secretion of eotaxin (p < 0.01) and RANTES (p < 0.01) in nasal polyp-derived fibroblasts. More importantly, this stimulatory effect was significantly suppressed by adding calcitriol (p < 0.002 for eotaxin and p < 0.008 for RANTES) or tacalcitol (p ≤ 0.009 for eotaxin and p ≤ 0.02 for RANTES). Therefore, the inhibitory effect of vitamin D derivatives on eotaxin and RANTES secretion might shed light not only on the disease mechanism, but also on the potential use of vitamin D in pharmacotherapy of Taiwanese patients with CRSwNP.

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Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is an inflammatory disease of the upper airway. Although its etiology and pathophysiology are still controversial, CRSwNP is believed to be the manifestation of complex inflammatory reactions [1]. Currently, topical steroids are the most commonly used therapeutic agents for CRSwNP, which are generally safe and well tolerated despite the inconsistent effects. Besides, other forms of anti-inflammatory and antiproliferative agents are being intensively evaluated [2]. However, the result is still unsatisfactory, and, because of frequent recurrence, repeated surgical interventions are often necessary.

Many cytokines and chemokines are thought to promote the pathogenesis of CRSwNP by facilitating the recruitment of inflammatory cells into the airway. Accumulation of eosinophils is one of the most characteristic features of CRSwNP. The degree of mucosal eosinophilia is correlated with more extensive disease and a decreased likelihood of surgical success [3,4]. Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) (CCL5) and eotaxin (CCL11) are two potent eosinophil-specific chemokines that contribute to chronic inflammation in CRSwNP. RANTES is a member of the C-C family of chemokines originally cloned from a subtracted cDNA library made from isolated RNA of a functional, nontransformed antigen-stimulated T cell line [5]. RANTES has been shown to induce cutaneous eosinophil infiltration when injected into dogs [6] and humans [7]. More importantly, there is a report that RANTES can induce eosinophil influx into the nasal mucosa [8]. The effects of RANTES on eosinophils include chemotaxis, induction of transendothelial migration and reactive oxygen species, and release of eosinophil cationic protein (ECP) [9,10]. By reviewing the literature, we found that RANTES immunoreactivity could be detected in nasal polyp specimens, and was localized predominantly to the epithelial [11] or endothelial cells [12]. Meyer et al. [13] speculated that RANTES played a key role in the mobilization of eosinophils into nasal polyp tissues. Moreover, Saji et al. [14] demonstrated the production of RANTES from nasal polyp fibroblasts after stimulation with IL-1β. It was suggested that IL-1β-induced production of RANTES in nasal polyp fibroblasts was necessary for eosinophil infiltration.

Eotaxin, also a member of the C-C family of chemokines, is a potent eosinophil attractant and can induce eosinophil recruitment and activation. It plays an indirect role in airway remodeling through recruitment of eosinophils and mast cells, which have profibrogenic and proangiogenic activities, into the site of inflammation [15]. Yao et al. [16] reported that eotaxin immunoreactivity was observed in most of the infiltrating eosinophils, among other eotaxin-positive inflammatory cells, and in epithelial and endothelial cells of nasal polyps. Furthermore, Yoshifuku et al. [17] showed that nasal polyp-derived fibroblasts could secrete eotaxin in the presence of proinflammatory mediators. Their findings suggested a critical effect of eotaxin on the selective recruitment of eosinophils in nasal polyps.

Vitamin D is known to regulate calcium and bone homeostasis. However, increasing recent evidence has revealed diverse physiological functions of vitamin D derivatives, including the immunomodulatory, anti-proliferative, and anti-inflammatory effects. Several studies have indicated the function of vitamin D in the pathogenesis and manifestation of airway diseases [18–20]. For example, vitamin D deficiency is related to the severity of asthma [18,21]. Maternal vitamin D intake from foods during pregnancy may be negatively associated with the risk of asthma and allergic rhinitis in childhood [22]. Furthermore, in cultured cell models, vitamin D derivatives could inhibit the proliferation of, and downregulate RANTES production in, nasal polyp-derived fibroblasts from Caucasian patients with CRSwNP [23,24]. Although there are several recent reports indicating that vitamin D deficiency is correlated with disease severity and acts as a major player in the immunopathology of CRSwNP [25–27], whether vitamin D might influence tissue eosinophilia of CRSwNP by modulating eotaxin and RANTES secretion especially in Asian patients is not clear and warrants further investigation.

Materials and methods

Patients and tissues

This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital, Kaohsiung, Taiwan (KMUH-IRB-990427), and written informed consent was obtained from each participant prior to the study. Based on the definition in European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2007 [28], patients with recent diagnosis of CRSwNP undergoing elective...
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endoscopic sinus surgery at our hospital were recruited. Patients with malignancies or asthma were excluded from the study. All patients were asked to stop antiinflammatory medications, including antibiotics and topical steroids, for at least 2 weeks prior to admission. The tissues were isolated from the polyps of CRSwNP patients during the operations. The tissue sections were fixed in formalin and stained with hematoxylin, and the number of eosinophil was counted to 200 × magnification under a light microscope. The definition of eosinophilic nasal polyps was similar to that described by Yoshifuku et al. [17]. In brief, five fields were examined for each section from which the average number [per high power field (HPF)] of eosinophil infiltration in the sample was determined. Those with >100 eosinophils/HPF and <10 eosinophils/HPF were considered as eosinophilic and noneosinophilic nasal polyps, respectively. Both eosinophilic and noneosinophilic polyp tissues were derived into primary fibroblast cultures, in which only the former was used for vitamin D treatment.

Reagents

IL-1β was purchased from Sigma-Aldrich (I9401; St. Louis, MO, USA). Calcitriol and tacalcitol were obtained from Tocris Bioscience (Ellisville, MO, USA). Human eotaxin and RANTES Quantikine ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA).

Primary culture of nasal polyp-derived fibroblasts

The obtained tissues were washed three times with antibiotics containing Hank’s Balanced Salt Solution (HBSS) and cut into small pieces (1 mm²). The tissue explants were treated with 0.5% trypsin for 7 minutes at 37°C, followed by neutralization with serum-containing Dulbecco’s modified Eagle’s medium (DMEM). After washing three times with HBSS, the explants were plated into 6-well culture dishes CellBIND Surface (product #3337, Corning, New York) and cultured with DMEM medium and with 10% FBS in a 37°C, 5% CO₂ humidified incubator for about 2 weeks. When the primary culture reached confluence at 70–80%, the nasal fibroblasts were subcultured into 10-cm dishes. Nasal fibroblasts at the third to eighth passages were used in the following experiments.

ELISA assays of eotaxin and RANTES

Nasal polyp-derived fibroblasts (5 × 10⁵) were cultured in each well of a 96-well plate with serum-containing or serum- and IL-1β-containing (10 ng/mL) medium for 24 hours. Subsequently, the medium of IL-1β-treated groups was replaced with fresh serum-free medium alone or together with calcitriol or tacalcitol (10μM), while the medium of mock-treated group was replaced with serum-free medium. One day later, the concentrations of secreted eotaxin and RANTES in the culture supernatants were determined by ELISA Human Eotaxin and RANTES Quantikine ELISA kit (R&D Systems, Minneapolis, MN) based on the manufacturer’s instructions. The optical density was read at 450 nm using a microplate reader, and the concentrations were calculated by interpolation from a standard curve. All experiments were done in triplicate and the results were expressed as mean ± SD.

Statistical analysis

The results of ELISA assays were analyzed by one-way analysis of variance (one-way ANOVA) followed by least significant difference (LSD) post hoc comparison of the treatments of mock versus IL-1β, IL-1β + calcitriol, and IL-1β + tacalcitol, in which p < 0.05 was considered statistically significant.

Results

Vitamin D inhibited IL-1β-induced secretion of eotaxin

To determine the potential therapeutic effect of vitamin D in alleviating tissue eosinophilia in nasal polyps, we examined whether calcitriol, an active form of vitamin D, and tacalcitol, a vitamin D derivative, could inhibit eotaxin secretion in nasal polyp-derived fibroblasts. Three independent cases of the fibroblast primary cultures from three patients with eosinophilic CRSwNP were established. Eotaxin was first induced by IL-1β (10 ng/mL) for 24 hours, followed by the treatment of calcitriol or tacalcitol (10µM) for another 24 hours. The levels of eotaxin in culture media were measured using ELISA assay. Analysis with one-way ANOVA indicated significant difference among the treatments (p < 0.001 in all cases). Further analysis with LSD post hoc comparison revealed significant differences between mock and IL-1β (16.82 ± 1.93 pg/mL vs. 39.24 ± 2.1 pg/mL in Case #1, p < 0.001; 21.85 ± 2.47 pg/mL vs. 47.41 ± 2.25 pg/mL in Case #2, p < 0.01; and 16.97 ± 0.98 pg/mL vs. 37.93 ± 2.34 pg/mL in Case #3, p < 0.001). This IL-1β-induced secretion of eotaxin was significantly suppressed with the addition of calcitriol (39.24 ± 2.1 pg/mL vs. 23.22 ± 1.48 pg/mL in Case #1, p < 0.001; 47.41 ± 2.25 pg/mL vs. 33.83 ± 0.92 pg/mL in Case #2, p = 0.002; and 37.93 ± 2.34 pg/mL vs. 23.15 ± 0.99 pg/mL in Case #3, p < 0.001) or tacalcitol (39.24 ± 2.1 pg/mL vs. 25.42 ± 1.27 pg/mL in Case #1, p < 0.001; 47.41 ± 2.25 pg/mL vs. 30.49 ± 1.58 pg/mL in Case #2, p = 0.009; and 37.93 ± 2.34 pg/mL vs. 23.47 ± 0.74 pg/mL in Case #3, p < 0.001; Fig. 1).

Vitamin D inhibited IL-1β-induced secretion of RANTES

We next investigated how vitamin D would influence RANTES secretion in nasal polyp-derived fibroblasts using the same approach. Analysis with one-way ANOVA on the ELISA results revealed significant difference among the treatments (p < 0.01 in all cases). Further analysis with LSD post hoc comparison showed that, in both primary cultures, RANTES was dramatically induced by IL-1β (32.47 ± 0.24 pg/mL vs. 54.69 ± 1.92 pg/mL in Case #1, p = 0.004; 34.29 ± 3.16 pg/mL vs. 56.68 ± 2.94 pg/mL in
such induction of RANTES was significantly suppressed in the presence of calcitriol (54.69 ± 1.92 pg/mL vs. 41.15 ± 1.6 pg/mL in Case #1, $p = 0.008$; 56.68 ± 2.94 pg/mL vs. 30.72 ± 3.83 pg/mL in Case #2, $p = 0.008$; and 26.47 ± 1.15 pg/mL vs. 16.85 ± 1.34 pg/mL in Case #3, $p < 0.001$) or tacalcitol (54.69 ± 1.92 pg/mL vs. 38.72 ± 1.84 pg/mL in Case #1, $p = 0.02$; 56.68 ± 2.94 pg/mL vs. 32.64 ± 2.63 pg/mL in Case #2, $p = 0.01$; and 26.47 ± 1.15 pg/mL vs. 16.61 ± 0.11 pg/mL in Case #3, $p < 0.001$; Fig. 2). Together, these results suggest that vitamin D can effectively inhibit eotaxin and RANTES secretion in nasal polyp-derived fibroblasts.

**Discussion**

Nasal polyposis remains one of the most challenging and recalcitrant diseases in clinical rhinology because of its complex etiology and predisposition to recurrence. However, it is well established that eotaxin and RANTES are central to tissue eosinophilia during nasal polyp formation [13,29,30]. We have previously shown that the serum level of 25-hydroxyvitamin D (25OHD), the major circulating form of vitamin D, in CRSwNP patients were significantly lower than those in chronic rhinosinusitis without nasal polyps (CRSsNP) patients [27]. Moreover, serum 25OHD level was significantly and inversely related to the size of nasal polyps. As vitamin D contains immunomodulatory effects on various immune cells [31,32], it is surmised that a low vitamin D level might be unable to attenuate cytokine release from inflammatory cells, leading to constitutive activation of inflammatory cascade in nasal polyps.

A previous study by Rostkowska-Nadolska et al. [23] demonstrated a significant dose-dependent decrease in the proliferation of nasal polyp-derived fibroblasts after the cells were treated with various doses of calcitriol and tacalcitol. Further investigation by the authors revealed that calcitriol and tacalcitol inhibited the synthesis of the proinflammatory cytokines IL-6 and IL-8 in the fibroblast cultures [33]. In this study, we hypothesized that vitamin D would affect tissue eosinophilia of Taiwanese patients with CRSwNP through regulating the production of eotaxin and RANTES. IL-1β is a cytokine with a wide spectrum of proinflammatory functions. In addition, recruitment of the inflammatory cells to the inflamed tissues involves a series of events including adhesion to endothelial cells,
transendothelial migration, and subsequent chemotactic movement. These processes are regulated through the release of inflammatory mediators and cytokines. However, eotaxin and RANTES are of particular significance for eosinophil recruitment and infiltration. Thus, we used IL-1β to induce eotaxin and RANTES production and asked whether such induction could be inhibited by vitamin D treatment in nasal polyp-derived fibroblasts. Indeed, the ELISA results showed that IL-1β could significantly induce the secretion of eotaxin and RANTES from the nasal fibroblasts, and this biological effect was significantly suppressed by the addition of calcitriol or tacalcitol. However, the mechanism behind the suppression of eotaxin and RANTES secretion by vitamin D is currently unknown. Whether vitamin D could block the intracellular synthesis of eotaxin and RANTES, either at the transcriptional or post-transcriptional level, or the release process of eotaxin and RANTES into the media deserves further study. Given that eotaxin and RANTES promote recruitment of eosinophils into the polyp microenvironment, the antiinflammatory effect of vitamin D against nasal polyps might be mediated through the inhibition of eotaxin and RANTES secretion. A more detailed study is ongoing.

Figure 2. Inhibition of IL-1β-induced RANTES secretion by vitamin D in nasal polyp-derived fibroblasts. Three cases (A, B, and C) of primary culture were treated with IL-1β alone, or IL-1β followed by calcitriol or tacalcitol. Secretion of RANTES into the culture media was measured by ELISA assay. Addition of the vitamin D derivatives resulted in decreased level of IL-1β-induced RANTES secretion. All experiments were done in triplicate and expressed as mean ± SD. A p value <0.05 represented statistical significance.

The definition of mucosal eosinophilia in CRSwNP is still debatable and could be population-dependent. Soler et al. [34] used a cutoff of more than five eosinophils/high power field (HPF) to define clinically relevant mucosal eosinophilia based on in vivo evidence of eosinophil activation. They also observed the impact of eosinophilia on quality-of-life outcomes at a higher density (>10 eosinophils/HPF) [35]. In this study, we employed a high standard of >100 eosinophils/HPF to define eosinophilic nasal polyps, ensuring the inducibility of eotaxin and RANTES in fibroblasts derived from such polyp tissues. Indeed, the three cases of fibroblast cultures used as the cell model were all derived from eosinophilic polyps, in which eotaxin and RANTES could be significantly induced by IL1-β. By contrast, these two chemokines could not be induced by IL1-β in fibroblasts derived from the polyp tissues of two noneosinophilic CRSwNP patients (Figures S1 and S2), indicating that vitamin D is a potential therapeutic agent specifically for eosinophilic nasal polyps. Interestingly, nasal polyps in the Asian population are suggested to be biased toward neutrophilic inflammation with less eosinophilia, in contrast to an eosinophil bias in the Belgian (white) population [36]. However, a recent study showed that plasma RANTES and
eotaxin levels were significantly elevated and correlated with disease severity in Taiwanese patients with CRSwNP [37]. The authors concluded that eotaxin and RANTES could play a role in enhancing eosinophil recruitment into the lamina propria of nasal polyps. Therefore, even though eosinophilia is less prominent in the Asian population with nasal polyps, eosinophils might still be essential for the pathogenesis of nasal polyps in Taiwanese patients. Based on our and others’ results, a possible mechanism for tissue eosinophilia in CRSwNP is that the proinflammatory cytokines induce eotaxin and RANTES secretion from nasal polyp fibroblasts, in turn selectively recruiting eosinophils from the circulation and resulting in eosinophilic infiltration and inflammation.

Our findings suggest that in patients with vitamin D deficiency, the inability of low vitamin D level to counter the secretion of eotaxin and RANTES might lead to chronic inflammation and eosinophilia characteristic of nasal polyps. Still, there are limitations in the present study. For example, the in vitro cell model might not completely reflect in vivo mechanisms. However, we thought that nasal polyp-derived fibroblasts are a legitimate in vitro model for this study for three reasons: (1) fibroblasts are an important component of the ground substances in nasal polyps and involved in a variety of inflammatory responses associated with disease pathogenesis [1]; (2) fibroblasts are one of the major stromal cells that produce and secrete eotaxin and RANTES; and (3) several published studies had used nasal polyp-derived fibroblasts as the cell model for tissue remodeling mechanism and therapeutic applications in nasal polyps [38,39].

In conclusion, this is the first study that investigates the effect of vitamin D on the production of eotaxin and RANTES in Taiwanese patients with CRSwNP. We showed that vitamin D derivatives could significantly inhibit eotaxin and RANTES secretion in nasal polyp-derived fibroblasts. These results may not only shed light on the mechanism of nasal polyp pathogenesis, but warrant further investigation for the clinical application of vitamin D in pharmacotherapy of CRSwNP.

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References


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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.kjms.2014.11.011.