

this unusual clinical presentation is necessary to appropriately diagnose and treat patients with cutaneous tertiary syphilis.

## CONFLICTS OF INTEREST

The authors have nothing to disclose.

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<https://doi.org/10.5021/ad.2018.30.6.751>



# Effects of Extracellular Calcium and Vitamin D on Cultured Human Sebocytes

Kyung Duck Park<sup>1</sup>, Jun Hong Park<sup>2</sup>, Mi Young Sohn<sup>2</sup>, Weon Ju Lee<sup>2</sup>

<sup>1</sup>Department of Dermatology, Chungnam National University School of Medicine, Daejeon, <sup>2</sup>Department of Dermatology, School of Medicine, Kyungpook National University, Kyungpook National University Hospital, Daegu, Korea

Dear Editor:

Calcium and 1,25-dihydroxyvitamin D<sub>3</sub> play important roles in epithelial cell functions. While low extracellular calcium levels in the culture medium induce rapid proliferation of keratinocytes, high extracellular calcium levels stimulate differentiation of keratinocytes. Vitamin D activates the proliferation of sebocytes and inhibits differentiation and sebum production in sebocytes<sup>1</sup>. In addition, vitamin D exerts an anti-inflammatory effect on sebocytes<sup>2</sup>. We evaluated the effects of extracellular calcium levels and the interaction between extracellular calcium levels and vitamin D on cultured human sebocytes.

Human sebocytes were cultured in Dulbecco's modified

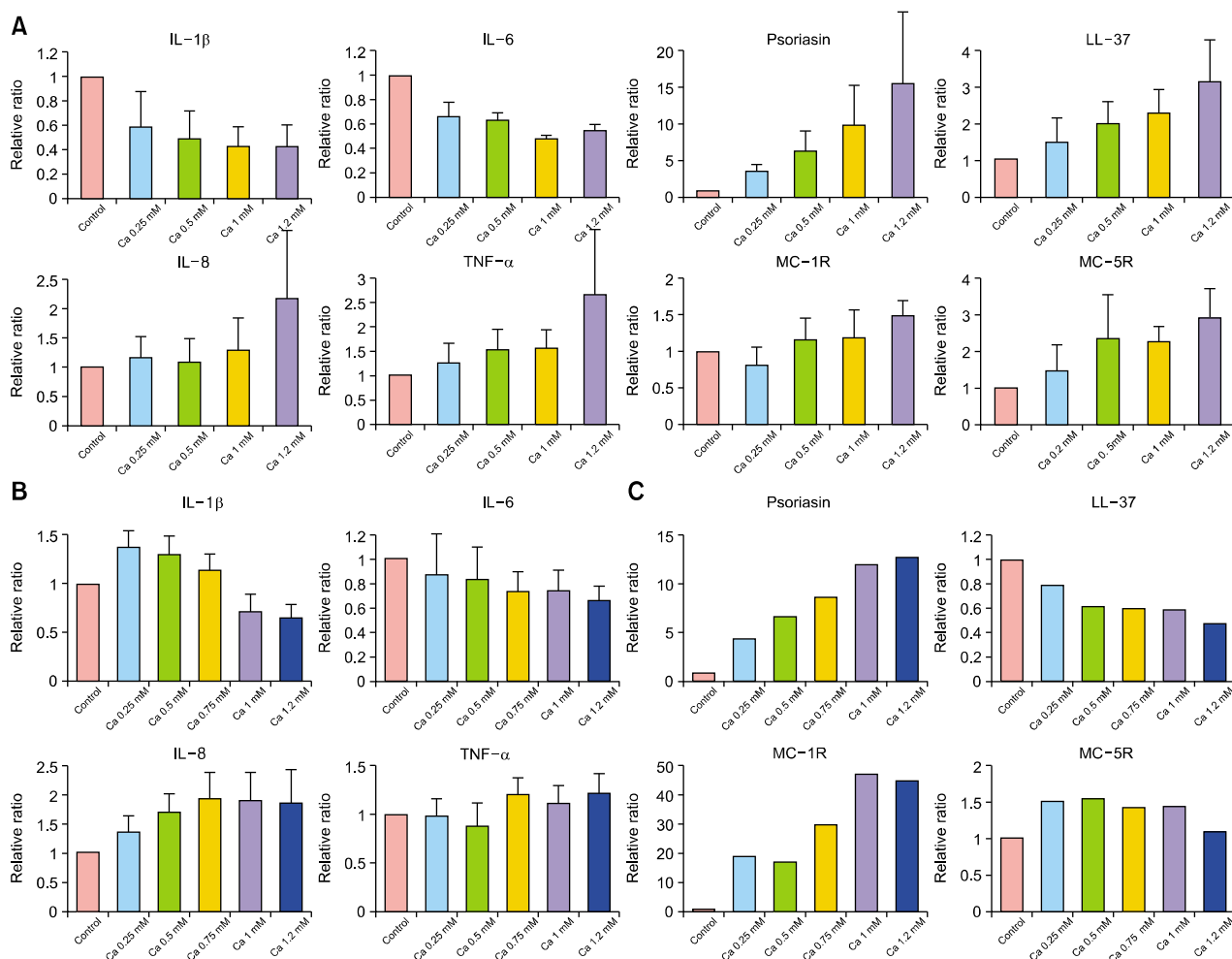
Eagle's medium (HyClone Laboratories, Logan, UT, USA) and Epilife (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The sebocytes were used for the experiment after their second subculture. The sebocytes were cultured in the presence of higher extracellular calcium levels (0.25, 0.5, 0.75, 1, or 1.25 mM). CCK8 analysis revealed the absence of any toxic effect of extracellular calcium at the concentrations tested on cell viability. The active form of vitamin D 10<sup>-6</sup> M was added into the culture media in the presence of each concentration of extracellular calcium. After the treatment of the sebocytes with vitamin D 10<sup>-6</sup> M in the presence of 0.25, 0.5, 0.75, 1, or 1.25 mM extracellular

Received October 31, 2017, Revised January 8, 2018, Accepted for publication January 12, 2018

**Corresponding author:** Weon Ju Lee, Department of Dermatology, Kyungpook National University Hospital, 130 Dongdeok-ro, Jung-gu, Daegu 41944, Korea. Tel: 82-53-420-5838, Fax: 82-53-426-0770, E-mail: weonju@knu.ac.kr  
ORCID: <https://orcid.org/0000-0001-5708-1305>

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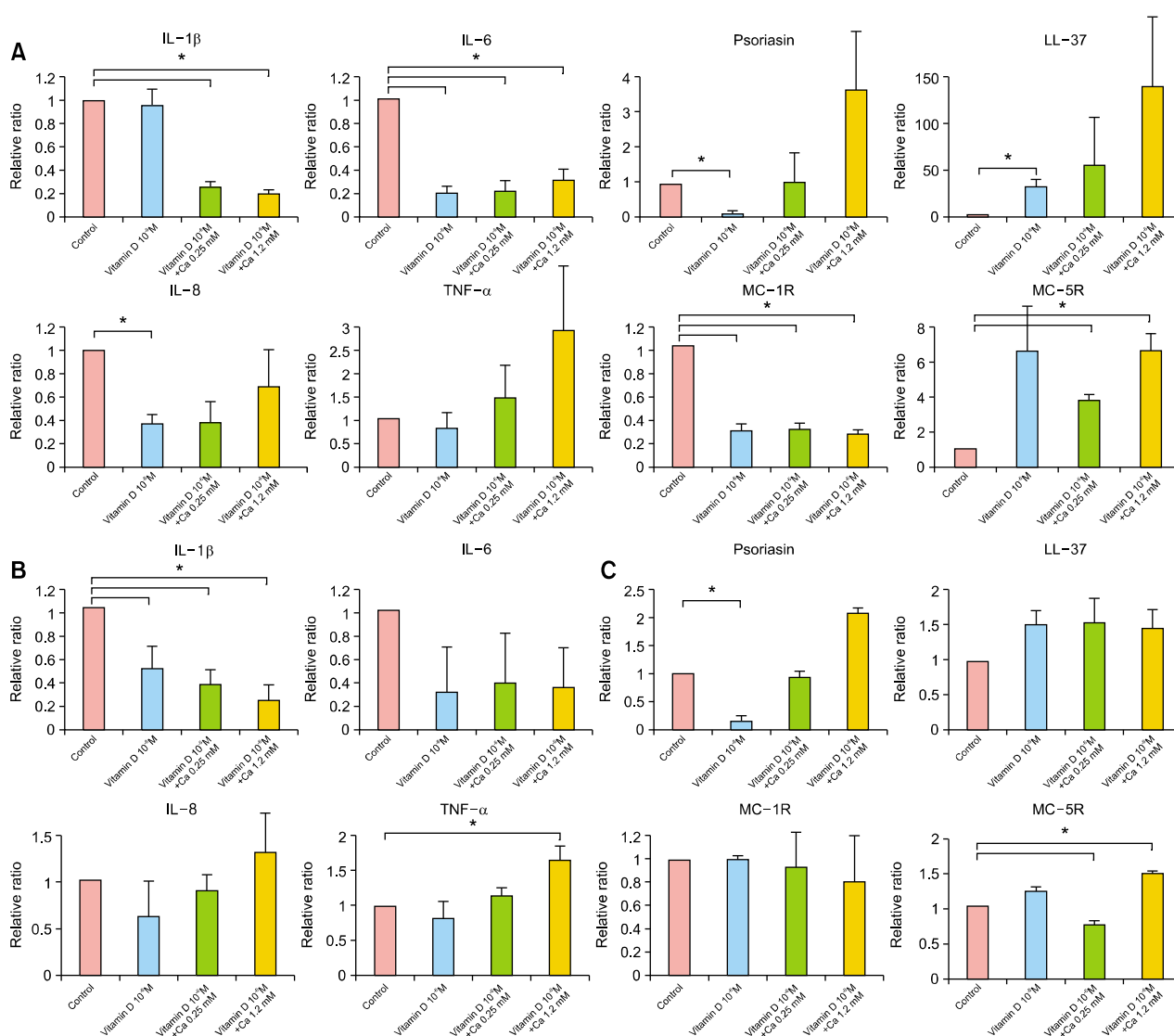


**Fig. 1.** (A) Gene expression of biomarkers in cultured human sebocytes after treatment with calcium (0.25, 0.5, 1, and 1.2 mM). (B) Protein expression of biomarkers in cultured human sebocytes after treatment with calcium (0.25, 0.5, 0.75, 1, and 1.2 mM) using ELISA. (C) Protein expression of biomarkers in cultured human sebocytes after treatment with calcium (0.25, 0.5, 0.75, 1, and 1.2 mM) using western blot analysis. IL: interleukin, TNF: tumor necrosis factor, MC-1R: melanocortin-1 receptor.

calcium, real-time PCR analysis was performed in triplicate with a LightCycler (Roche Diagnostics, Indianapolis, IN, USA). The expression of biomarkers, such as interleukin (IL)-1  $\beta$ , IL-6, IL-8, and tumor necrosis factor (TNF)- $\alpha$ , in the sebocytes was measured in triplicate using ELISA (R&D Systems, Shanghai, China). Western blot analysis was performed to evaluate the expression of biomarkers, including psoriasis, LL-37, melanocortin-1 receptor (MC-1R), and MC-5R, in the sebocytes. Data were statistically analyzed with paired two-tailed Student's t-test using the statistical product and service solutions software (IBM Co., Armonk, NY, USA) for Windows (version 18). Data were presented as relative mean values  $\pm$  standard deviation. A value of  $p < 0.05$  was considered as statistically significant.

Higher extracellular calcium levels stimulated differentiation of sebocytes. The higher levels of extracellular

calcium decreased the expression of IL-1  $\beta$  and IL-6 in gene and protein levels in the sebocytes (Fig. 1A, B). Gene and protein expression levels of IL-8, TNF- $\alpha$ , psoriasis, MC-1R, and MC-5R increased in the sebocytes in the presence of higher levels of extracellular calcium in a concentration-dependent manner (Fig. 1). In addition, higher extracellular calcium levels showed a concentration-dependent increase in the expression of LL-37 gene in the sebocytes (Fig. 1A). The treatment of the sebocytes with vitamin D resulted in a decrease in gene and protein expression of IL-1  $\beta$  ( $p < 0.05$ ), IL-6 ( $p < 0.05$ ), IL-8 ( $p < 0.05$ ), TNF- $\alpha$ , psoriasis ( $p < 0.05$ ) and MC-1R (Fig. 2). On the other hand, gene and protein expression of LL-37 ( $p < 0.05$ ) and MC-5R in the sebocytes increased following their incubation with vitamin D (Fig. 2A, C). In the presence of higher concentrations of extracellular calcium, vitamin D treatment downregulated the expression of IL-1  $\beta$



**Fig. 2.** (A) Gene expression of biomarkers in cultured human sebocytes after treatment with vitamin D ( $10^{-6}$  M) and calcium (0.25 or 1.2 mM). (B) Protein expression of biomarkers in cultured human sebocytes after treatment with vitamin D ( $10^{-6}$  M) and calcium (0.25 or 1.2 mM) using ELISA. (C) Protein expression of biomarkers in cultured human sebocytes after treatment with vitamin D ( $10^{-6}$  M) and calcium (0.25 or 1.2 mM) using western blot analysis. IL: interleukin, TNF: tumor necrosis factor, MC-1R: melanocortin-1 receptor. \* $p < 0.05$ .

( $p < 0.05$ ), IL-6 ( $p < 0.05$ ), IL-8 and MC-1R ( $p < 0.05$ ), but not TNF- $\alpha$  ( $p < 0.05$ ) and psoriasis, in the sebocytes in gene and protein levels (Fig. 2). The treatment of the sebocytes with vitamin D in the presence of higher concentrations of extracellular calcium resulted in upregulation of LL-37, and MC-5R ( $p < 0.05$ ) expression in gene and protein levels (Fig. 2A, C).

The epidermal calcium gradient induces proliferation of basal keratinocytes, while it mediates differentiation of keratinocytes from the upper epidermal layers at higher extracellular calcium concentrations<sup>3</sup>. Higher extracellular calcium concentrations elevate the levels of intracellular

free calcium. Vitamin D regulates cells expressing vitamin D receptors, such as keratinocytes. Some physiologic functions of vitamin D are associated with calcium. Vitamin D increases the intracellular calcium levels in keratinocytes, leading to their differentiation<sup>4</sup>. Therefore, both calcium and vitamin D can regulate the differentiation of epidermal keratinocytes. However, vitamin D inhibits the differentiation and sebum production in sebocytes<sup>5</sup>. Vitamin D may suppress sebocyte differentiation by binding to vitamin D receptor-retinoid X receptor dimers in a calcium-independent manner<sup>6</sup>. Like keratinocytes, higher extracellular calcium levels stimulated differentiation of

cultured human sebocytes in this study. On the contrary, Zouboulis et al.<sup>7</sup> reported that the increased extracellular calcium level stimulated proliferation of immortalized human SZ95 sebocytes but decreased extracellular calcium level induced differentiation of the sebocytes. Differentiation of sebocytes at higher extracellular calcium levels can be explained by the increase in the intracellular calcium levels and  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH)<sup>8</sup>.  $\alpha$ -MSH not only increases intracellular calcium levels, but also stimulates MC-1R expression and lipid synthesis in immortalized sebocytes<sup>8</sup>. We also observed in this study that MC-1R and MC-5R were overexpressed in cultured human sebocytes at higher extracellular calcium levels. The differentiated sebocytes at higher extracellular calcium levels showed the increased expression of other biomarkers, such as IL-8, TNF- $\alpha$ , and psoriasis. As known previously, vitamin D inhibited the expression of IL-8, TNF- $\alpha$ , and psoriasis as well as IL-1  $\beta$ , IL-6, and MC-1R in cultured human sebocytes in this study. In addition, vitamin D inhibited the expression of IL-8 and MC-1R in cultured human sebocytes at higher extracellular calcium levels. However, the increased expression of TNF- $\alpha$  and psoriasis in the sebocytes was not inhibited by vitamin D. Like sebocytes in this study, keratinocytes has been known to induce psoriasis at higher calcium levels<sup>9</sup>. Furthermore, it was reported that TNF- $\alpha$  can be related to stimulate the expression of antimicrobial peptides<sup>10</sup>. In conclusion, extracellular calcium and vitamin D exert a regulatory function on the expression of biomarkers in cultured human sebocytes.

## CONFLICTS OF INTEREST

The authors have nothing to disclose.

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