視黃酸對牙周韌帶細胞上的表皮生長因子感受器之調節

Regulation of epidermal growth factor receptor expression on periodontal ligament cell by retinoic acid

計畫類別：個別型計畫
計畫編號： NSC89-2314-B-039-014
執行期限：88 **年** 8 **月** 1 **日 至** 89 **年** 7 **月** 31 **日**
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計畫參與人員： 蔡小雲、簡華宏

一 中文摘要

在視黃酸對牙周韌帶細胞的影響研究中，我們使用 RT-PCR **的方法結果顯示視黃酸會影響骨細胞標記，如鹼性磷酸脢，**osteocalcin **和** osteopontin **在人類牙周韌帶細胞上的表現，於數種被測試的訊息核糖核酸**(mRNA)**中視黃酸對鹼性磷酸脢有最顯著的影響。鹼性磷酸脢之基因表現隨著視黃酸濃度之增加而減少。我們的結果支持視黃酸可以抑制人類牙周韌帶細胞的分化之理論，而它的抑制機轉很可能是和表皮生長因子一樣，靠著調節牙周韌帶細胞上的表皮生長因子感受器來抑制牙周韌帶細胞的分化。**

關鍵詞 ： **視黃酸**, **鹼性磷酸脢**, **人類牙周韌帶細胞**

Abstract

The effect of retinoic acid on the regulation of human periodontal ligament (PDL) fibroblast differentiation was investigated. RT-PCR analysis of human PDL cells showed that the expression of osteoblast markers such as alkaline phosphatase, osteocalcin and osteopontin were all affected by retinoic acid. The expression of the osteoblast marker alkaline phosphatase was the most significantly decreased among the three proteins tested at an increased concentration of retinoic acid. Our results strongly suggested that retinoic acid is capable of down-regulation of the human PDL cell differentiation into mineralized tissue forming cells. The mechanism of inhibitory effect of retinoic acid on PDL cell differentiation may involve in regulation of EGF-receptor expression.

Keywords: retinoic acid, alkaline phosphatase, human periodontal ligament (PDL) cell

二 緣由與目的 (Background and Purpose)

Periodontitis is known as a chronic inflammatory disease associated with

putative periodontal pathogens found in the dental plaque at disease sites. The disease is characterized by the progressive destruction of connective tissue attachment to the tooth and resorption of the alveolar bone that subsequently results in the loss of tooth (Robertson 1992). The microorganisms appear to incite destruction of the supporting tissues directly by releasing their lysosomal enzymes and endotoxins, and indirectly by activation of host cells to produce a variety of biologically active substances, such as arachidonic acid metabolites, cytokines and proteolytic enzymes (Socransky and Haffajee, 1991; Genco 1992). The optimal goal of periodontal therapy involves not only the resolution of inflammatory lesion in the periodontal tissues and the arrest of periodontal disease progression, but also the regeneration of lost periodontium. In this aspect, periodontal ligament (PDL) fibroblasts likely play a central integrative role in achieving periodontal regeneration.

The PDL has been regarded as the source of the precursor cells for osteoblasts and cementoblasts which are capable of restoring the lost alveolar bone and cementum (Beertsen *et al.*, 1997). Therefore, the rapid repopulation of the PDL cells to undergo either differentiation into osteoblasts or remain undifferentiated status to maintain its own width and structural integrity is critical for full periodontal regeneration. In order to achieve full periodontal regeneration, it is important to illustrate the regulatory mechanisms responsible for PDL progenitor cells to either differentiate into osteoblast or remain as an undifferentiated phenotype for self-renewal.

Retinoic acid has been reported as a potent regulator of cell proliferation (Mason *et al.*, 1985) and differentiation (Moore *et al.*, 1984). Our preliminary data reveals that the treatment of rat PDL fibroblasts with retinoic acid increases the synthesis of EGF-receptor that has been shown to be expressed only on undifferentiated preosteoblasts and prechodrocytes. This observation suggests that retionic acid may play an important role in maintaining PDL fibroblastic cells in an undifferentiated state. Since PDL fibroblasts are known to have osteoblast-like characteristics, we therefore propose that retinoic acid may act as a negative regulator for human PDL cell differentiation via modulation of EGF-receptor expression. In this proposed study, we were unable to detect the expression of EGF-receptor in human PDL, although we were able to detect it in rat PDL in our preliminary results. Considering not to spend time in rat but in human PDL, we have changed the direction to investigate the effect of retinoic acid on osteoblast markers, such as alkaline phosphatase, osteocalcin and osteopontin in human PDL. The effect of EGF on the regulation of PDL cell differentiation are also explored.

The proposed study will help to clarify the effect of retinoic acid in human PDL cell differentiation as proposed previously. By understanding the negative regulatory

mechanism of PDL cell differentiation and how the PDL progenitor cell remains as an undifferentiated phenotype, we may develop molecular based strategies to first recruit and promote mitogenic growth of PDL cells and subsequently increase mineralized tissues (possibly bone and cementum) forming activity via the cell cycle.

三 Material and Methods

RNA isolation

Human PDL cells (a free gift from Dr. Cho, State University of New York at Buffalo) were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics to 80%-90% confluence at 37 °C in a 5% CO_2 incubator. Cells were then starved overnight in DMEM with 1% fetal bovine serum before the treatment of cells with a serial concentration of retinoic acid (10^{-5} 10^{-6}, 10^{-7}, 10^{-8} M) for 24 hours. Total cellular RNA was isolated by guanidinium isothiocyanate extraction using TRIzol reagent (GIBCO/BRL) according to the manufacture's instructions. After determination of the appropriate concentration of retinoic acid for treatment of PDL cell, the cells were then treated with this concentration (10^{-6} M) for up to 24 hours for a time course study.

RT-PCR analysis

Reverse-transcription of mRNA in the total RNA preparation was performed by using MMLV(H^-) reverse transcriptase (Promega). Approximately 4 μg of total RNA and 1μg of oligo-dT (15mer, serve as a primer) were used in the reverse transcription reaction. cDNA synthesis from RNA was conducted in parallel and the cDNA quality checked first by gel electrophoresis and by subsequent PCR amplification using GAPDH primers prior to use in any further studies. The cDNA products were then PCR amplified using approximately 10 ng of cDNA template and different primer sets. The followings are primers selected for analyzing different bone marker proteins: For analyzing human osteocalcin (Bilbe *et al.* , 1996), forward primer: ATGAGAGCCCTCACACTCCTC, reverse primer: CTAGACCGGGCCGTAGAAG-CG; For analyzing human osteopontin (Rickard *et al.*,1996), forward primer: CCAAGTAAGTCCAACGAAAG, reverse primer: GGTGATGTCCTCGTCTGTA; For analyzing human alkaline phosphatase (Rickard *et al.*,1996), forward primer: ACGTGGCTAAGAATGTCATC, reverse primer, CTGGTAGGCGATGTCCTTA; For analyzing human GAPDH, forward primer: GGTGAAGGTCGGAGTCAACGG, reverse primer, GGTCATGAGTCCTTCCACGAT (Rickard *et al.*,1996). Primers (EGFR-3) for analyzing human EGF-receptor were obtained commercially from Clontech company. The sequences of EGFR-3 primers are as following: forward primer, CTACCACCACTCTTTGAACTGGACCAAGG, reverse primer, TCTATGCTCTCACCCCGTTCCAAGTATCG. PCR products were then analyzed on

1% agarose gels.

Northern Blot Analysis

RNA samples (10-20 μg/lane) were electrophoresed on a 1% agarose gel containing formaldehyde, and then transferred and fixed to the Nytran Plus membrane (Schleicher and Schuell) according to the manufacture's protocols. The cDNA probe was labeled with fluorescein-N^6-dATP (NEN) instead of [^{32}P]-dCTP.

四. Results and Discussion

Analysis of the effect of retinoic acid on expression of various bone marker proteins on the human PDL cells.

RT-PCR analysis of human PDL cells showed that the expression of alkaline phosphatase, osteocalcin and osteopontin were all affected by the treatment of the cells by retinoic acid. The expression of alkaline phosphatase (ALP) was decreased at an increase of retinoic acid concentration. At highest concentrations of 10^{-5}M, almost no amplification of alkaline phosphatase was detected under our analyzing system. The expression of osteopontin (OPN) and osteocalcin (OC) was slightly affected by retinoic acid treatment only at a high concentration, 10^{-5}M. Since ALP and OC are known as osteoblast specific markers. The decreased expression of these proteins after the retinoic acid treatment of the human PDL cells further suggested that retinoic acid down-regulate the PDL osteoprogenitor cell differentiation. Although OPN is not a osteoblast-specific marker, the decrease of OPN expression after retinoic acid treatment may also suggest the down-regulating effect of retinoic acid on PDL cells. The above described observation is consistent with our previous preliminary report using retinoic acid treated rat PDL cell. Previously, the treatment of rat periodontal ligament fibroblasts with retinoic acid increases the synthesis of EGF-receptor that has been shown to be expressed only on undifferentiated preosteoblasts and prechodrocytes. In our study, we also tested the expression of EGF-receptor on human PDL fibroblast; however, both northern analysis using fluorescent cDNA probe and RT-PCR analysis using EGFR-3 primers did not show any expression of EGF-receptor on human PDL cells. In conclusion, our observations suggested that retinoic acid down-regulate the human PDL osteoprogenitor cell differentiation. Therefore, retinoic acid may play an important role in maintaining the human PDL in an undifferentiated state and possibly promote the proliferation of PDL fibroblasts.

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