

INDUCTION OF TYPE I COLLAGEN AND OSTEOCALCIN IN HUMAN DENTAL PULP CELLS BY RETINOIC ACID

Sroisiri Thaweboon¹, Boonyanit Thaweboon¹, Suwan Choonharuangdej¹, Panjit Chunhabundit² and Prapan Suppakatana²

¹Department of Microbiology, ²Department of Anatomy, Faculty of Dentistry, Mahidol University, Bangkok, Thailand

Abstract. Retinoic acid has been known to play a key role in the regulation of bone cell differentiation and function. The effects of retinoic acid on human dental pulp cells, which contain several characteristics similar to those of bone cells, has yet to be elucidated extensively. The effects of retinoic acid on human dental pulp cells in terms of type I collagen and osteocalcin induction were investigated *in vitro*. Dental pulp cells obtained from the teeth of young patients (age between 18-22 years) were cultured and subsequently treated with various concentrations of retinoic acid (0, 10⁻⁷, 10⁻⁶, 10⁻⁵ M) in serum-free DMEM. At different time intervals (8, 12 and 24 hours), the levels of type I collagen and osteocalcin secreted were determined using Type I Procollagen C-Peptide and Gla-type Osteocalcin EIA kits, respectively. Induction effects were evaluated using analysis of variance and the Duncan's multiple rank test. Retinoic acid at concentrations of 10⁻⁵, 10⁻⁶, 10⁻⁷ M was able to induce type I collagen and osteocalcin production in human dental pulp cells within 12 hours of exposure. Dose-dependent induction was observed only after 24 hours. A two-fold increase in osteocalcin level was detected after exposed to 10⁻⁵ M retinoic acid within 24 hours. Our data suggest that retinoic acid at concentrations of 10⁻⁵, 10⁻⁶, 10⁻⁷ M has the ability to induce type I collagen and osteocalcin secretions in human dental pulp cells *in vitro*.

INTRODUCTION

Teeth are composed of three calcified tissues, enamel, dentin, and cementum, and one delicate specialized connective tissue, the pulp. Of the three calcified tissues, the dentin forms the bulk of the tooth substance and gives the basic shape to each tooth. The inorganic content of dentin is composed of apatite molecules, chiefly in the form of hydroxyapatite crystals. Although having a considerably high organic content, dentin is a harder tissue than bone. Type I collagen is the major organic component found in dentin. These fibers not only provide a matrix for the deposition of hydroxyapatite crystals, but also limit the quantity of mineral that can be deposited in the dentin. Osteocalcin, a non-collagenous matrix protein, is also involved in crystal formation and the growth of mineralized tis-

ues of dentin. In bone, osteocalcin is a biochemical marker indicating the metabolism and turnover of such tissue. Several previous studies have suggested the roles of osteocalcin in bone resorption, osteoclast differentiation or crystal formation and growth (Boskey *et al*, 1985; Romberg *et al*, 1986; Hunter and Goldberg, 1994). Additionally, recent studies indicate that osteocalcin may play other roles in bone formation and remodeling (Ducy *et al*, 1996). Both osteocalcin and type I collagen secreted by dental pulp fibroblasts are therefore believed to play a regulatory role in dentin formation.

Retinoic acid, an active metabolite of vitamin A, can promote growth and differentiation of many cell types. Vitamin A has effects on the development of epithelial cells and bone as well as the maintenance of epithelial cells. Nizel and Papas (1989) observed the cessation of bone growth, abnormal bone length and remodeling sequences in young animals with vitamin A deficiency. The effect of vitamin A deficiency on teeth has also been elucidated. Vitamin A deficiency has been reported to influence odontogenesis through atrophy and metaplasia of the enamel

Correspondence: Sroisiri Thaweboon, Department of Microbiology, Faculty of Dentistry, Mahidol University, 6 Yothi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2644-8644-6 ext 4811-2; Fax: 66 (0) 2644-8634
E-mail: dtstw@mahidol.ac.th

organ, as well as to induce atrophy of odontoblasts resulting in atypical dentin formation of incisor teeth in experimental animals (McDowell *et al*, 1987).

In rat dental pulp cell cultures, retinoic acid was shown to stimulate mRNA expression and the synthesis of osteopontin, which is a non-collagenous protein associated with dentinogenesis and regulation of dental pulp mineralization (Ohishi *et al*, 1999). The objective of this study was to investigate the effects of retinoic acid on human dental pulp cells in terms of type I collagen and osteocalcin secretions.

MATERIALS AND METHODS

Cell culture

Human dental pulp cells were obtained from teeth extracted from young patients (age 18-22 year). Cells were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotics, including 10,000 units/ml of penicillin G and 25 µg/ml of amphotericin B as

fungisone. All the components employed were the products of Gibco BRL, USA. Cultures were grown at 37°C in an incubator supplied with 5% CO₂. The 3rd to 6th passages of human dental pulp cells were used in the experiment.

Retinoic acid and treatment

Retinoic acid (Sigma) was solubilized with ethanol to obtain a concentration of 5 x 10⁻³ M, and stored in the dark at -20°C until used. Soluble retinoic acid was serially diluted with serum-free DMEM to 10⁻⁵, 10⁻⁶ and 10⁻⁷M. The addition of retinoic acid did not affect the pH of the culture medium. Dental pulp cells were seeded into 24-well plates at a density of 1 x 10⁴ cells/well, and incubated for 5-6 days until confluency. The cells were washed with PBS three times before serum-free DMEM containing different concentrations of retinoic acid (0, 10⁻⁷, 10⁻⁶, 10⁻⁵ M) were added.

Type I collagen and osteocalcin secretion measurement

Culture supernatant was collected at different time intervals at 8, 12 and 24 hours. The

Table 1
Effect of retinoic acid on type I collagen secretion in human dental pulp cells.

Exposure Time (h)	Type I collagen (ng/10 ³ cells)			
	control	retinoic acid(M)		
		10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
8	12.84±2.05	16.68±1.33	14.72±2.01	10.40±3.11
12	15.97±1.44	25.17±2.32 ^a	22.66±0.78 ^a	19.03±2.60 ^a
24	23.32±1.81	36.18±3.25 ^a	31.51±1.09 ^a	26.87±1.38 ^a

The results are shown as means ± SEM (n=4). ^ap-value<0.05, significantly different from the control.

Table 2
Effect of retinoic acid on osteocalcin secretion in human dental pulp cells.

Exposure Time (h)	Osteocalcin (ng/10 ⁴ cells)			
	control	retinoic acid(M)		
		10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
8	3.06±0.31	3.22±0.10	3.08±0.14	3.15±0.27
12	3.32±0.05	4.87±0.22 ^a	4.71±0.03 ^a	4.65±0.15 ^a
24	5.65±0.11	10.93±0.28 ^a	9.76±0.09 ^a	7.09±0.42 ^a

The results are shown as means ± SEM (n=4). ^ap-value<0.05, significantly different from the control.

levels of secreted type I collagen and osteocalcin were determined using a Type I Procollagen C-Peptide EIA kit (Takara Shuzou, Japan) and a Osteocalcin EIA kit (Takara Shuzou, Japan); respectively.

Statistical analysis

The effect of retinoic acid on cells was evaluated by analysis of variance and Duncan's multiple rank test for statistical significance of the differences.

RESULTS

Retinoic acid, at the concentrations of 10^{-7} , 10^{-6} and 10^{-5} M, was found to induce both type I collagen and osteocalcin secretion from dental pulp cells between 12 and 24 hours of exposure, as is depicted in Tables 1 and 2. Dose-dependent induction of type I collagen and osteocalcin levels was observed only after 24 hours of treatment. Marked increases in the components were detected in cultures treated with 10^{-5} M retinoic acid. At this concentration, a two-fold increase in the level of osteocalcin was found at 24 hours of exposure.

DISCUSSION

Human dental pulp cells have been shown to contain several characteristics similar to those of osteoblastic cells. Such characteristics include the presence of alkaline phosphatase activity, the ability to produce type I collagen, and non-collagenous proteins associated with mineralization, such as osteocalcin and osteonectin. In addition, these cells are capable of differentiating into odontoblast-like cells to form dentin. Under normal physiological conditions, primary odontoblasts in dental pulp are regulated to produce new dentin at a very slow rate. Once the primary odontoblasts are damaged by noxious stimuli, such as bacteria or their by-products in carious lesions, the dead cells can be replaced by the odontoblast-like cells differentiated from dental pulp cells that produce new dentin matrix, termed as reparative dentin. This increases the thickness of the hard tissue barrier overlying the pulp. During the reparative dentinogenesis, collagen synthesis is accelerated and dentin proteins are then deposited within the extracellular

matrix before mineralization occurs.

The present study examined the secretion of type I collagen and osteocalcin in response to retinoic acid. Treatment with retinoic acid led to an increase in the secretion of type I collagen and osteocalcin from dental pulp cells. Our results are in agreement with the previous studies of osteoblastic cells (Health *et al*, 1989; Oliva *et al*, 1993; Aubin and Liu, 1996). Retinoic acid has been reported to upregulate osteocalcin gene expression in bone cells. An increased synthesis of osteocalcin mRNA, and their contemporaneous presence results in an amplified gene transcription (Oliva *et al*, 1993; Jimenez *et al*, 2003). Moreover, two studies based on the use of plasmid constructs reported the interaction of retinoic acid with the osteocalcin promoter (Morrison *et al*, 1989; Schule *et al*, 1990). A molecular-based hypothesis has been postulated to explain the inductive effect of retinoic acid on osteocalcin; there might be a responsive element (s) for retinoic acid receptors (RARE) present on the osteocalcin gene. The present findings reveal that retinoic acid affects osteocalcin production not only in osteoblastic cells, but also in dental pulp cells. Bloch-Zupan *et al* (1994) and Berkovitz *et al* (1993) have found retinoic acid nuclear receptors and two retinoic acid-binding proteins in murine dental pulp. These findings support the idea that retinoic acid has an effect on dental pulp functions. Regarding the involvement in type I collagen, retinoic acid has important effects on collagen homeostasis. It has been shown to increase type I collagen expression in rat immature pre-osteoblastic cells (Health *et al*, 1989), quail chondrocytes (Sanchez *et al*, 1993) and human skin fibroblasts (Geesin *et al*, 1990). In contrast, it had inhibitory effects on human osteoblastic cells (Mahonen *et al*, 1998; Ogston *et al*, 2002). The discrepancy may be from the degree of cellular differentiation, donor species, dose and duration of exposure. Increased osteocalcin and type I collagen in our study indicate that retinoic acid may prove to be useful in designing reparative treatments in dental applications.

In summary, this study confirms that retinoic acid can promote dental pulp cell differentiation and may facilitate the repair process through the

induction of type I collagen and osteocalcin secretion. Further investigations are required to obtain more useful information on the regulation of a target gene implicated in this induction, beneficial for *in vivo* study designs.

ACKNOWLEDGEMENTS

The authors wish to thank Assoc Prof Woralluck Prachayabreud for her valuable advice and encouragement. This study was supported by a grant from the Faculty of Dentistry, Mahidol University.

REFERENCES

- Aubin JE, Liu F. The osteoblast lineage. In: Bilezikian J, Raisz L, Rodan G, eds. Principles of bone biology. San Diego: Academic Press, 1996: 51-68.
- Berkovitz BK, Maden M, Eriksson U. This distribution of cellular retinoic acid-binding protein I during odontogenesis in the rat incisor. *Arch Oral Biol* 1993; 38: 837-43.
- Bloch-Zupan A, Mark MP, Weber B, Ruch JV. *In vitro* effects of retinoic acid on mouse incisor development. *Arch Oral Biol* 1994; 39: 891-900.
- Boskey AL, Wians FH, Hauschka PV. The effect of osteocalcin on in-vitro lipid-induced hydroxyapatite formation and seeded hydroxyapatite growth. *Calcif Tissue Int* 1985; 37: 57-62.
- Ducy P, Desbois C, Boyce B, *et al*. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996; 382: 448-52.
- Geesin JC, Gordon JS, Berg RA. Retinoids affect collagen synthesis through inhibition of ascorbate-induced lipid peroxidation in cultured human dermal fibroblasts. *Arch Biochem Biophys* 1990; 278: 350-5.
- Health J, Rodan S, Yoon K, Rodan GA. Rat clavicular cell lines immortalised with SV-40 large T antigen: constitutive and retinoic acid induced expression of osteoblastic features. *Endocrinology* 1989; 124: 3060-8.
- Hunter GK, Goldberg HA. Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. *Biochem J* 1994; 302: 175-9.
- Jimenez MJ, Balbin M, Alvarez J, *et al*. A regulatory cascade involving retinoic acid, Cbfa1, and matrix metalloproteinases is coupled to the development of a process of perichondrial invasion and osteogenic differentiation during bone formation. *J Cell Biol* 2001; 155: 1333-44.
- Mahonen A, Jukkola A, Risteli J, Maenpaa PH. Type I procollagen synthesis is regulated by steroids and related hormones in human osteosarcoma cells. *J Cell Biochem* 1998; 68: 151-63.
- McDowell EM, Shores RL, Sprangler EE, Wenk ML, De Luca LM. Anomalous growth of rat incisor teeth during chronic intermittent vitamin A deficiency. *J Nutr* 1987; 117: 1265-74.
- Morrison NA, Shine J, Fragonas JC, Verkest V, MaMenemy ML, Eisman JA. 1, 25-dihydroxyvitamin D-repressive element and glucocorticoid repression in the osteocalcin gene. *Science* 1989; 246: 1158-61.
- Nizel AE, Papas AS. Nutrition in clinical dentistry. Philadelphia: WB Saunders, 1989.
- Ogston N, Harrison AJ, Cheung HFJ, Ashton BA, Hampson G. Dexamethasone and retinoic acid differentially regulate growth and differentiation in an immortalised human clonal bone marrow stromal cell line with osteoblastic characteristics. *Steroids* 2002; 67: 895-906.
- Ohishi M, Horibe M, Ikedo D, *et al*. Effect of retinoic acid on osteopontin expression in rat clonal dental pulp cells. *J Endodon* 1999; 25: 683-5.
- Oliva A, Rgione FD, Fratta M, Marrone G, Palumbo R, Zappia V. Effect of retinoic acid on osteocalcin gene expression in human osteoblasts. *Biochem Biophys Res Com* 1993; 191: 908-14.
- Romberg RW, Werness PG, Riggs BL, Mann KG. Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium binding proteins. *Biochemistry* 1986; 25: 1176-80.
- Sanchez M, Gionti E, Arcella A, Pontarelli G, De Lorenzo F. Alpha 2 (1) collagen gene expression is up-regulated in guail chondrocytes pretreated with retinoic acid. *Biochem J* 1993; 295: 115-9.
- Schule R, Umesonon K, Mangelsdorf DJ, Bolado J, Pike JW, Evans RM. Jun-Fos and receptors for vitamin A and D recognize a common response element in the human osteocalcin gene. *Cell* 1990; 61: 497-504.