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In vitro effects of ascorbic acid and β-glycerophosphate on human gingival fibroblast cells

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ABSTRACT

Ascorbic acid (AA) and β-glycerophosphate (βG) are considered *in vitro* osteogenic factors important to the differentiation of osteoblastic progenitor and dental pulp cells into mineralized tissue-forming cells. So, the present study investigated *in vitro* if these mineralizing inducible factors (AA and βG) could influence differentiation of human gingival fibroblasts when compared with human pulp cells and osteogenic cells derived from rat calvaria cultured. The expression of osteopontin (OPN) and osteoadherin (OSAD) was analyzed by indirect immunofluorescence, immunocytochemistry as well as Western-blotting. In addition, the main ultrastructural aspects were also investigated. No mineralized matrix formation occurred on gingival fibroblasts induced with AA + βG. On these cells, no expression of OPN and OSAD was observed when compared with pulp cells, pulp cells induced with AA + βG as well as osteogenic cells. Ultrastructure analysis additionally showed that gingival fibroblasts exhibited typical fibroblast morphology with no nodule formation. The present findings showed that AA and βG could not promote a mineralized cell differentiation of human gingival fibroblasts and confirm that human dental pulp cells, as the osteogenic cells, are capable to form a mineralized extracellular.

1. Introduction

The dental pulp consists of soft connective tissue surrounded by the peripheral odontoblast layer, responsible for dentin mineralized matrix formation. The mechanism involved in the differentiation of dental pulp cells into odontoblasts is not well established when an injury occurs, producing reparative dentin (Ten Cate, 1998; Arana-Chavez and Massa, 2004).

Although well demonstrated, the presence of undifferentiated cells in dental pulp known as dental pulp stem cells (DPSCs) (Gronthos et al., 2000), fibroblasts are also a considerable source for the differentiation of mineralized tissue-forming cells (Yamamura, 1985). Pulpal fibroblasts exhibit some specialities in relation to fibroblasts present in other connective tissues such as the expression of tenascin, osteonectin, osteocalcin, bone sialoprotein, alkaline phosphatase and dentin sialophosphoprotein, all of them mineralized tissue-related extracellular matrix proteins (Gronthos et al., 2000; Martinez et al., 2000; Martinez and Araújo, 2004; Laino et al., 2006).

It is known that a wide variety of extracellular matrix proteins are involved in mineral deposition. OPN and OSAD are proteins which have affinity for the nucleation of the mineral phase, crystal growth and mineral maturation (Hunter et al., 1996; Sodek and McKee, 2000). Several studies have demonstrated the expression of OPN in mineralized tissues (Hunter et al., 1996), mainly in the bone matrix (Giachelli and Steitz, 2000) which is involved in the interaction between minerals and bone cells. It is secreted by pre-osteoblast, osteocytes, osteoblasts and "odontoblast-like" cells (Butler, 1989; McKee and Nanci, 1996; Aguiar and Arana-Chavez, 2007). Expression of OSAD, originally isolated from mineralized bone matrix (Wendel et al., 1998), is observed not only on osteoblasts, which have an important role in regulating the mineralization of mineralized tissues (Sommarin et al., 1998; Shen et al., 1999; Rehn et al., 2008), as well as in odontoblasts (Buchaille et al., 2000; Petersson et al., 2003).

In vitro studies show that supplementation of culture medium with ascorbic acid, sodium glycerophosphate (Kasugai et al., 1988; Nakashima, 1991; Kuo et al., 1992; Couble et al., 2000; Zhang et al., 2005) and dexamethasone (Kasugai et al., 1993) induce pulp cells to differentiate into "odontoblast-like" cells and therefore the formation of a mineralized extracellular matrix (Kasugai et al., 1988; Nakashima, 1991; Couble et al., 2000). In addition, they are considered osteogenic factors important to the differentiation of osteoblastic progenitor cells in cell culture (Irie et al., 1998; Gartland et al., 2005). However, the effect of these factors in

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nonosteogenic cells such as human gingival fibroblasts has not yet been studied.

The purpose of this *in vitro* study was to verify whether these mineralizing inducible factors [ascorbic acid (AA) and β-glycerophosphate (βG)] could influence differentiation of human gingiva fibroblasts when compared with human pulp cells and osteogenic cells derived from rat calvaria cultured, by evaluating the immunoexpression of osteopontin and osteoadherin by indirect immunofluorescence, immunocytochemistry as well as Western-blotting. In addition, the main ultrastructural aspects were also examined.

2. Materials and methods

2.1. Cell isolation and primary cultures

The gingival mucosa fibroblasts were obtained from explants of healthy attached human gingival from patients submitted to aesthetic periodontal surgery and, the pulpal cells, from a human third molar germ extracted for orthodontic reasons. These tissues were used with the patient's informed consent and appreciation of the Research Ethics Committee of the University of São Paulo (Protocol # 601) from three different donors.

The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 1% antimicotic solution (Sigma), containing 10% foetal calf serum (Invitrogen, Burlington, ON, Canada). The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2, supervised every 24 h. When the cells reached subconfluence, they were harvested with trypsin and subcultured and used in the following experiments.

The osteogenic cells were obtained according to research protocols approved by the Ethical Committee for Animal Research of the University of São Paulo, Brazil (Protocol # 86/05). Osteogenic cells were isolated by sequential trypsin/collagenase digestion of calvaria bone from newborn (2–4 days) Wistar rats, as previously described (De Oliveira et al., 2003).

The obtained cells were cultured in α-minimum essential medium (α-MEM) (Invitrogen, Burlington, ON, Canada) containing 10% foetal bovine serum (FBS, Cultilab, Campinas, Brazil) and 1% gentamicin (Invitrogen), plated in 60-mm diameter plastic culture dishes and incubated at a density of 110 cells/mm^2.

After 24 h, for mineralization induction, the medium was supplemented with 10 mM β-glycerophosphate (Sigma®) and 50 μg/ml of ascorbic acid (Invitrogen®) (Nakashima, 1991; Kuo et al., 1992; Couble et al., 2000) in all cell primary cultures.

In order to certify the osteogenic phenotype, calcium and phosphate-based mineral deposits were evaluated by Alizarin-red and von Kossa staining, respectively (Bonewald et al., 2003) (Fig. 1).

Table 1
Primary antibodies.

Antibody	Host	Source
Anti-osteopontin (OPN) (monoclonal) (MPIIIB10-1)	Mouse	DSHB[a]
Anti-human osteoadherin (OSAD) (polyclonal)	Rabbit	Couble et al. (2004)[b]

[a] Developmental Studies Hybridoma Bank.
[b] The antibody OSAD was kindly supplied by Prof. Dr. Françoise Bleicher (Université Claude-Bernard Lyon-4, France).

2.2. Indirect immunofluorescence

To evaluate the immunoexpression of mineralized matrix proteins on the induced cell cultures, cells grown on coverslips were fixed in methanol for 6 min at 4 °C, rinsed in PBS followed by blocking with 5% skimmed milk for 30 min at room temperature. The primary antibodies are described at Table 1. Control staining reaction was performed using PBS as non-immune IgGs at the same dilution used for primary antibody. The coverslips were rinsed in PBS and anti-rabbit or anti-mouse IgGs coupled to fluorescein (Vector Laboratories Inc., Burlingame, CA, USA) were used as secondary antibody. The coverslips were rinsed in PBS and in distilled water, and mounted in Vectashield (Vector®).

The expression of the studied proteins was carried out using a Zeiss Axiophot 2 conventional fluorescence microscope (Zeiss, Carl Zeiss MicroImaging, Oberköchen, Germany) equipped with

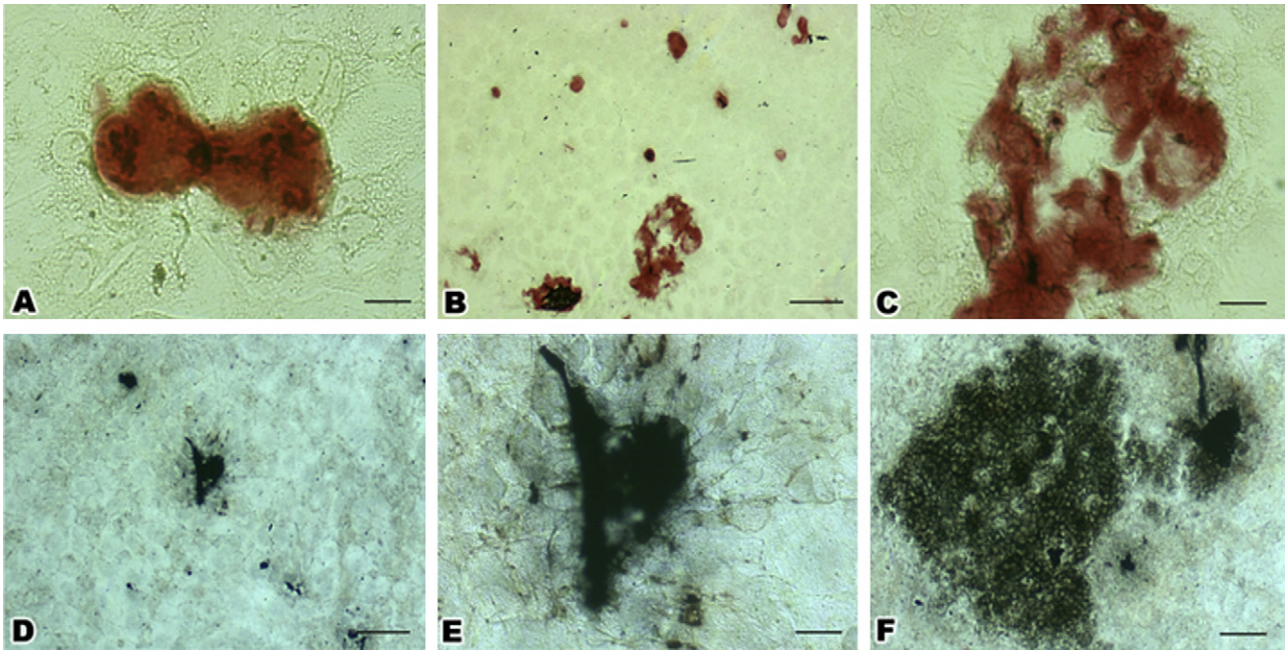

Fig. 1. Alizarin-red (A–C) and von-Kossa (D–F) staining in calvaria-derived osteogenic cell cultures after 14 days of treatment with AA + βGP. Bone-like nodule formation was evidenced in these cell cultures in the presence of AA + βGP. Bars: A, C, E, F = 20 μm; B, D = 100 μm.