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Osteoblast-like properties of human periodontal ligament cells: an *in vitro* analysis

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SUMMARY Identifying the biological properties of the cells residing within the periodontal ligament (PDL) will help in understanding the role that these cells play in the various functions of the periodontal ligament, and will improve the success of clinical procedures such as orthodontic tooth movement. For this purpose, fibroblasts isolated from human periodontium were cultured and characterized both histochemically and biochemically with respect to their putative osteoblast-like properties.

Histochemically, cultured PDL fibroblasts showed an intense staining for alkaline phosphatase (ALP). Biochemically, the basal ALP activity increased in culture over time. ALP levels after stimulation with 1α,25-dihydroxyvitamin D_3 were significantly higher than those of control cultures. Moreover, immunofluorescence against osteocalcin (a highly reliable osteoblastic marker) was strongly positive. Von Kossa staining of the cell cultures revealed the formation of mineral-like nodules. These results indicate that human PDL fibroblasts exhibit *in vitro* phenotypic characteristics consistent with osteoblast-like cells, thus suggesting that such cells have the potential to differentiate into osteoblasts and/or cementoblasts.

Introduction

The periodontal ligament (PDL) is a pluropotential structure. Primarily it anchors teeth in the jaws, but it fulfils several other functions such as tooth eruption, physiological mobility during mastication, and physiological or induced alveolar bone remodelling.

It is suggested that while the tooth and ligament is fundamentally contained by either the maxillary or mandibular bones, the source of bone cells initially lining the tooth socket is, in fact, separate and distinct from the surrounding bone (Tenenbaum, 1992). It is well known that after tooth extraction the alveolar bone resorbs. Moreover, implants inserted as tooth replacements become ankylosed in the bones and cannot be moved orthodontically. The reasons for the above phenomena remain obscure. It seems that the apparent lack of the PDL might be the cause.

It is suggested that the PDL might be the source of bone-forming cells (osteoblasts). *In vivo* studies have shown that the periodontal ligament contains osteoprogenitor cells (Roberts and Weiss, 1974; Gould *et al.*, 1980; Roberts and Morey, 1985; Roberts *et al.*, 1987). These cells most likely go through a maturation scheme from small fibroblastic precursor cells to osteoprogenitor cells, as they develop into bone-forming cells (McCulloch and Melcher, 1982, 1983).

In vitro, the osteogenic potential of cells is characterized by, among others, their levels of alkaline phosphatase (ALP), the induction of ALP in response to 1α,25-dihydroxyvitamin D_3 and parathyroid hormone (PTH), the production of non-collagenous bone-associated proteins, and the initiation of mineralized loci. Studies have shown that PDL cells exhibit ALP activity (Kawase *et al.*, 1886; Piche *et al.*, 1989), and this activity is higher when compared with gingival fibroblasts (Somerman *et al.*, 1988). Bovine and rat PDL possess osteogenic properties (Nojima *et al.*, 1990; Cho *et al.*, 1992).

The biological properties of the cells residing within the PDL, and especially their putative osteoblastic potential, is of pivotal importance in order to understand the biological basis of many

clinical strategies, such as force application and subsequent bone remodelling during orthodontic tooth movement.

In the present study, human PDL cells were analysed and their osteoblastic properties were evaluated histochemically and biochemically. Specifically, in an attempt to provide more definite answers with regard to the nature of human PDL cells, human PDL fibroblasts were characterized with respect to their ALP activity under physiological and stimulated conditions, their reaction against osteocalcin (a highly reliable osteoblastic marker), and their ability to initiate mineralization nodules *in vitro*.

Materials and methods

Isolation and culture of PDL fibroblasts

The donors of the PDL tissues were young healthy individuals. PDL fibroblasts were obtained from the roots of healthy extracted third molars. Biopsies were placed onto 35 mm culture dishes with 1 ml culture medium, Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Paisley, Scotland) supplemented with 1 per cent antibiotics (penicillin G, streptomycin, amphotericin B; Gibco BRL, Grand Island, NY) and 10 per cent fetal calf serum (FCS; Biochrom, Seromed, Berlin, Germany). Incubations were performed in a humid environment of 5 per cent CO_2/95 per cent air at 37°C. Outgrowth of the cells was first observed after 10 days in culture. Cell subcultures proceeded by trypsin–EDTA digestion, washing, and replanting. Following trypsin digestion of the primary cultures, cells were seeded in duplicate onto 35 mm culture dishes at a density of 2.5×10^4 cells per dish. PDL fibroblasts exhibited strong positive immuno- fluorescence against vimentin. At day 3, two dishes were used to obtain cell counts up to day 14 and the doubling time of the cells was calculated.

Alkaline phosphatase staining

Localization of ALP was studied using cells cultured on plastic culture dishes (Falcon, Becton and Dickinson, Plymouth, UK). Cells were fixed in neutral buffered formalin and washed in distilled water. Localization of ALP was demonstrated by incubation with sodium-α-naphthol phosphate as substrate in the presence of Fast Red Violet LB salt.

Assay for basal and hormonally induced ALP activity

PDL fibroblasts were plated in 35 mm culture dishes (3×10^5 cells per dish). At day 5, basal ALP levels started to be measured using a modified kinetic method (Sigma 245 kit, St Louis, MO), each time in duplicate dishes. The amount of *p*-nitrophenol liberated was determined by a spectrophotometer at 405 nm.

To examine the effect of 1α,25-dihydroxyvitamin D_3, PDL fibroblasts were seeded onto 35 mm culture dishes at a density of 3×10^5 cells per dish with 8 ml DMEM supplemented with 10 per cent FCS and 1 per cent antibiotics. On the fifth day, the medium was removed, and after rinsing with phosphate-buffered saline (PBS) the cell cultures were incubated in 5 ml DMEM supplemented with 0.2 per cent bovine serum albumin (BSA) and 10^{-8} M 1α,25-dihydroxyvitamin D_3 (kindly provided by LaRoche Laboratories, Basel, Switzerland) or in control medium (i.e. DMEM, 0.2 per cent BSA). Following a 48 hour incubation at 37°C under standard cell culture conditions, the medium was removed and the cultures were rinsed with PBS. Basal ALP activity and ALP activity with and without 1α,25-dihydroxyvitamin D_3 induction was measured every day. A parallel set of duplicate PDL cell cultures (at the same initial cell density) was used to count cells every day. Differences between ALP values with and without vitamin D_3 induction were analysed by Student's *t*-test.

Osteocalcin immunofluorescence

PDL fibroblasts cultured in 35 mm culture dishes in the presence of 1α,25-dihydroxyvitamin D_3 as described above, were used for osteocalcin immunofluorescence. After 48 hour incubation with 1α,25-dihydroxyvitamin D_3 at 37°C under standard cell culture conditions, the medium was removed and the cultures were washed three times with 2 ml PBS. Cell cultures were fixed with 2 per cent formaldehyde for 20 minutes. Following three sequential washes with PBS, cell cultures were incubated with the first antibody, anti-human osteocalcin (Paesel &

Lorei 40495, Frankfurt, Germany), overnight at 4°C. Cell cultures were washed with PBS and incubated with the second antibody, anti-rabbit IgG (Paesel & Lorei 1716), for 2 hours at 4°C. After rinsing with PBS, the cell culture were microscopically observed and photographed.

Mineralization

PDL fibroblasts were plated in 35 mm culture dishes (3×10^5 cells/plate) and cultured in DMEM containing 10 per cent FCS and 1 per cent antibiotics, and supplemented with 50 mg/ml ascorbic acid (Roth 3525, Karlsruhe, Germany), 10 mM sodium β-glycerophosphate (Sigma G9891) and 10^{-7} M dexamethasone (stock solution in ethanol; Sigma D1756) or in control medium (i.e. DMEM, FCS, antibiotics and 0.1 per cent ethanol). The cells were fed every other day. Between the third and fourth week, the cells were fixed with methanol and stained by the Von Kossa method for light microscopy.

Results

Isolation and culture of PDL fibroblasts

PDL cell cultures grew at the logarithmic growth phase with doubling times of 20.2 hours. As they approached confluency, the cells formed an even monolayer with parallel orientation of fusiform cells (Figure 1). Hyperconfluent cell cultures formed multilayered colonies of randomly orientated cells.

ALP staining

As shown in Figure 2, more than 85 per cent of a confluent PDL cell culture was stained positively for ALP.

Assay for basal and hormonally induced ALP activity

In the PDL cell cultures, basal ALP activity was detected on day 3 and continued to increase markedly day by day in culture. The osteoblast-like character of these cells was further investigated by examining their response to 1α,25-dihydroxyvitamin D_3. Incubation of PDL cells with 1α,25-dihydroxyvitamin D_3 resulted in progressive increases in ALP activity, while control cultures (no vitamin D_3) showed only a slight increase. The differences between the two groups were statistically significant for every measurement except that on day 7.

Figure 1 Confluent human PDL fibroblast cultures (original ×63).

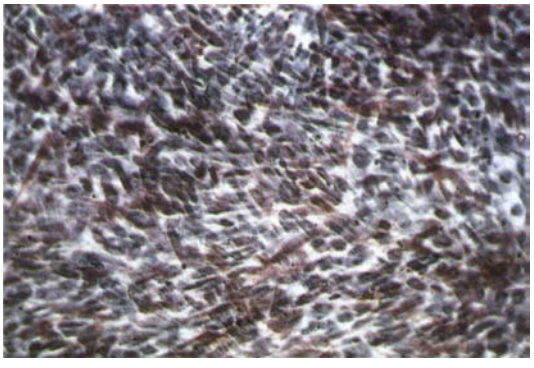

Figure 2 Strong positive ALP staining of human PDL fibroblasts (original ×100).

Figure 3 shows the time course of changes in basal and induced ALP activity with reference to cell numbers. Results represent mean values from four experiments.

Osteocalcin immunofluorescence

Microscopic observation of the PDL cell cultures revealed a strong positive reaction against osteocalcin (Figure 4).

Mineralization

After 3 weeks in culture, the cells produced mineral deposits that could be seen by using a light microscope alone (Figure 5a). Following Von Kossa staining, these mineral deposits could be observed as amorphous brown–black precipitates (Figure 5b). This silver staining

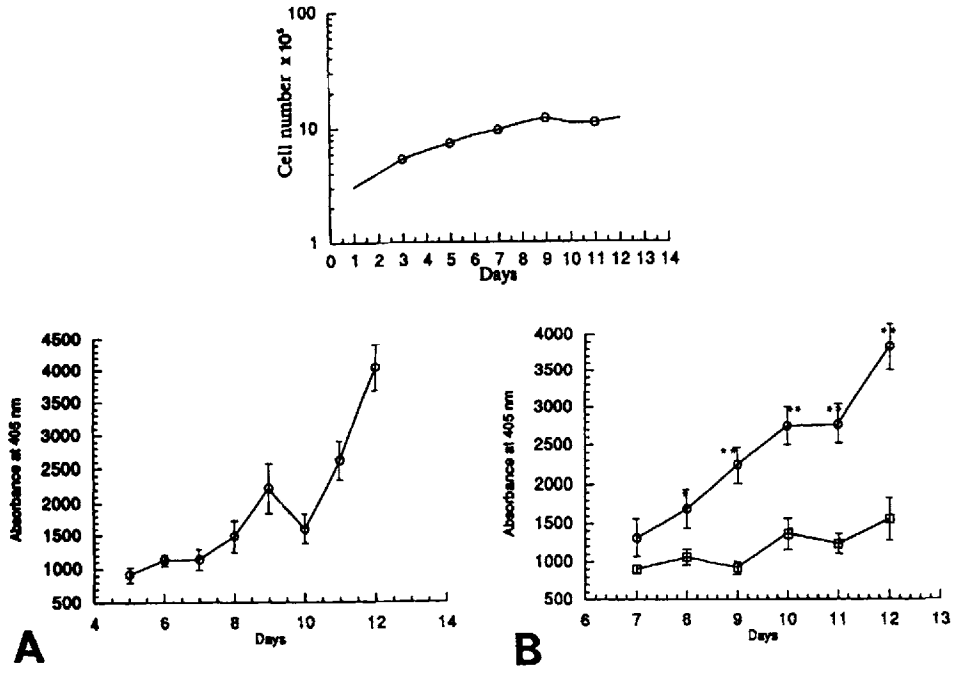

Figure 3 Levels of ALP in human PDL cultures. The absorbance was measured at 405 nm and in relation to cell number. (A) Basal ALP activity measured in culture. Note the gradual elevation of the basal ALP activity with time. (B) ALP activity with and without the addition of 1α,25-dihydroxyvitamin D_3. A sharp elevation in ALP activity after 48 hours of vitamin D_3 stimulation can be seen. Each point and error bar represent a mean value with standard deviation for four experiments. Values indicated by * and ** were significantly different ($P < 0.05$) from those of untreated cell cultures.

reaction is indicative of calcium phosphates. Since hydroxyapatite, a calcium phosphate derivative, is a main constituent of calcified tissues, the method has empirically proven to be very reliable and sensitive for qualitative (but not quantitative) demonstration of the presence of the mineral phase. These precipitates were prominent in areas of high cell and extracellular matrix concentration.

Discussion

In the present study, several osteoblast-like phenotypic characteristics have been identified *in vitro* for cells isolated from human periodontium including: (a) high basal ALP activity, (b) markedly increased ALP activity after vitamin D_3 induction, (c) vitamin D_3-dependent production of osteocalcin and (d) initiation of mineral-like nodules in the presence of a supportive medium.

ALP is an enzyme closely associated with the mineralization process. It has been suggested that hydrolysis of monophosphate esters by the enzyme produces a high concentration of phosphates (Beersten and van den Bos, 1989), leading to supersaturation and subsequent

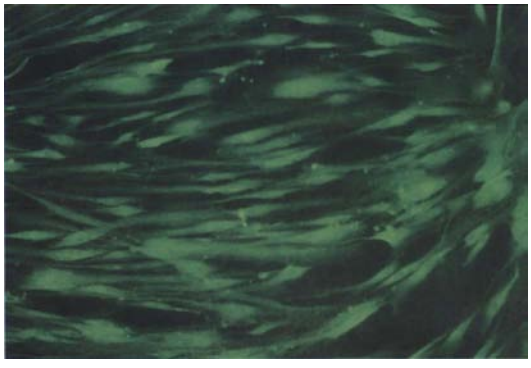

Figure 4 Human PDL fibroblasts show strong positive immunofluorescence reaction against osteocalcin (original ×250).

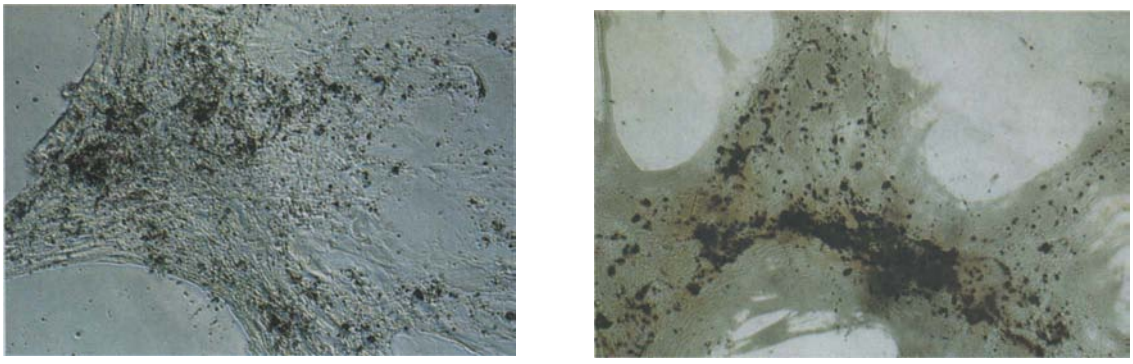

Figure 5 (a) Light microscopy of PDL fibroblasts after 3 weeks in culture (original ×63). (b) Von Kossa staining of the PDL cell cultures revealing amorphous brown–black precipitates in close association with high cell concentrations (original ×63).

precipitation of calcium phosphate salts in the collagenous substrate (Robison, 1923). In this study, confluent PDL cell cultures exhibited high ALP activity histochemically and biochemically. Intense ALP activity has been reported in the PDL tissues and cells of different species (Kawase *et al.*, 1986; Yamashita *et al.*, 1987; Piche *et al.*, 1989), and more recently it has been shown that the enzyme activity is higher adjacent to cellular compared with acellular cementum (Groeneveld *et al.*, 1995). Although the term ALP is broad and describes a group of enzymes which have the capacity to remove phosphate moieties from organic substrates at an alkaline pH, Nojima *et al.* (1990) showed that ALP in bovine PDL cells is the bone-specific isoenzyme.

ALP activity was significantly augmented after induction with 1α,25-dihydroxyvitamin D_3 which is the most biologically active metabolite of vitamin D_3 and is regarded as an important regulator of calcium homeostasis (Norman *et al.*, 1984). In human bone cells, 1α,25-dihydroxyvitamin D_3 at doses of 5×10^{-6} M inhibits cell proliferation (Skjodt *et al.*, 1985). Bone-derived cell populations have been found to increase their ALP levels significantly following exposure to 1α,25-dihydroxyvitamin D_3 (Manolagas *et al.*, 1981; Beresford *et al.*, 1986).

The vitamin D_3-dependent production of osteocalcin by PDL cells was examined immunohistochemically. Also known as the bone-Gla protein, osteocalcin is distributed to cells of calcified tissues including dentine and subcutaneous calcifications in rats. Osteocalcin production and secretion appear to be regulated by 1α,25-dihydroxyvitamin D_3, and there is some speculation that it is important for the recruitment of progenitor cells in regions of bone resorption (Simmons and Grynpas, 1992). Vitamin D_3-dependent production of osteocalcin is a property of virtually all primary osteoblasts, clonal osteoblast lines, and osteosarcoma cells in culture. Osteocalcin appears to be a 'late' marker during osteoblastic differentiation, characteristic of more mature osteoblasts and the ROS 17/2.8 osteosarcoma line (Hauska, 1992). In the present analysis, human PDL cells exhibited a strong positive reaction for osteocalcin after stimulation with 1α,25-dihydroxyvitamin D_3. The antibody used is specific for the intact osteocalcin molecule, with the recognition site located at the carboxy terminus, and detects proteins of the human, bovine, and monkey species. Bovine PDL cells synthesize a protein immunologically cross-reactive with bovine osteocalcin (Nojima *et al.*, 1990). Osteocalcin has not been described as a normal characteristic of PDL fibroblasts. Bronckers *et al.* (1994) have shown that although osteocalcin is present in bone cells, this molecule cannot be detected in rat PDL fibroblasts. A recent study has shown that cells positive for osteocalcin mRNA, in a rat maxillary dentoalveolar unit, were not only osteoblasts and osteoprogenitor cells, but osteocalcin was seen along the root surface (Takano-Yamamoto *et al.*, 1994). This raises the possibility that our cell culture might be a cementoblast culture, but

as no specific cell markers exist to distinguish cementoblasts from osteoblasts, this remains a matter of speculation.

Furthermore, the formation of mineral-like nodules at the third to fourth week was detected in PDL cell cultures. The fibroblasts were cultured in the presence of ascorbic acid, β-glycerophosphate, and dexamethasone. *In vitro*, this 'supplemented' medium with glucocorticoids appears to be stimulatory on bone-cell metabolism parameters associated with bone formation. It has been found that dexamethasone stimulates osteogenesis and mineralization in cultured folded embryonic chick periostea (Tenenbaum and Heersche, 1985). Moreover, it has been shown (Bellows *et al.*, 1986, 1987) that glucocorticoids increase the number of bone nodules appearing in cultures of fetal rat calvarial cell populations cultured under the same conditions as in this study. The mineralization nodules which we observed were associated with high densities of cells producing extracellular matrices. Transmission electron microscopic studies revealed that these mineralization nodules were in the direct neighbourhood of striated collagen fibres (data not shown). Cho *et al.* (1992) suggested that the morphological characteristics of the *in vitro* formed mineralization nodules in rat PDL are different from those of bone, and they speculated that they might represent another type of mineralized tissue, possibly acellular cement.

Conclusions

It has clearly been shown in this investigation that cells residing within the periodontal ligament have phenotypes characteristic of osteoblast-like cells. Whether these cells further differentiate to osteoprogenitor cells and ultimately to osteoblasts and/or cementoblasts, and what factors influence this process, remain an unresolved issue. Further research should focus on identifying the factors that play a role in this differentiation pathway. Moreover, the putative role that mechanical forces (physiological or induced; Basdra *et al.*, 1995, 1996a,b) exert during this process should be explored in order to elucidate further the mechanisms operating during the different functions of the periodontal ligament.

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