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Maturation-state dependent response of human periodontal ligament cells to an intermittent parathyroid hormone exposure *in vitro*

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Background and objectives: Parathyroid hormone (parathyroid hormone) has been shown to be capable of exerting anabolic effects on bone when administered intermittently. We hypothesized that parathyroid hormone will modulate the response of periodontal ligament cells in terms of anabolic effects with respect to proliferation, differentiation and the production of key regulatory factors of bone remodeling such as osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) in a maturation-state dependent manner.

Methods: Periodontal ligament cells were cultured from human bicuspids obtained from six patients. Following characterization, confluent and preconfluent periodontal ligament cells were challenged with parathyroid hormone (1–34) for 0, 1, 3, 6 or 24 h within three incubation cycles of 48 h each. At harvest, the cell number, alkaline phosphatase specific activity and osteocalcin, osteoprotegerin and RANKL production were determined by means of semiquantitative polymerase chain reaction (PCR) and immunoassays. Dermal fibroblasts and MG63 osteoblast-like cells served as a reference.

Results: Intermittent parathyroid hormone treatment of confluent periodontal ligament cells caused a significant increase in proliferation (+32% maximum) whereas alkaline phosphatase activity, osteocalcin and osteoprotegerin decreased at the transcriptional and translational level (−59.7% maximum). In preconfluent periodontal ligament cells, parathyroid hormone induced a decrease in proliferation (−66.3% maximum) but an increase in differentiation and osteoprotegerin production (+49.2% maximum). RANKL was hardly detectable and unaffected by parathyroid hormone treatment. Similar results were obtained in MG63 cells, whereas parathyroid hormone stimulation did not alter any of the parameters examined in dermal fibroblasts.

Conclusion: These results indicate that human periodontal ligament cells respond to an intermittent parathyroid hormone exposure with changes in proliferation, differentiation and osteoprotegerin production in a maturation-state dependent manner and therefore might be regulatorily involved in periodontal regeneration.

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Parathyroid hormone is a polypeptide hormone involved in the regulation of bone remodeling and is essential to the maintenance of calcium and phosphate homeostasis (1). Recently, it has been shown in several *in vivo* and *in vitro* studies that parathyroid hormone is capable of modulating the response of bone cells in terms of catabolic or anabolic actions (2) depending on the time course and duration of its application (3, 4). Whereas catabolic effects of parathyroid hormone have been assigned to continuous parathyroid hormone exposure (5), anabolic effects accompanied by bone formation have been observed following intermittent parathyroid hormone challenge in humans, as well as in various animal and osteoblast cell culture models (6, 7). Although the exact molecular mechanisms that mediate the effects of parathyroid hormone remain to be elucidated, these responses have partially been attributed to osteoblast precursor proliferation, mature osteoblast function (8), activation of various anabolic growth factors, such as components of the insulin-like growth factor system, e.g. IGF-I, IGF-II, as well as IGF binding proteins and receptors (9), and also to changes in the expression and production of local factors that have been discovered to be key regulatory molecules in bone remodeling, such as osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) (10, 11). Furthermore, an inhibition of apoptosis in osteoblasts in response to intermittent parathyroid hormone treatment has been reported (12, 13). These findings have been transferred to the clinic and proved beneficial to osteoporotic women who displayed an increase in bone mass following intermittent subcutaneous parathyroid hormone injections (14).

As mentioned above, the RANK/RANKL/osteoprotegerin system plays a crucial role in the regulation of osteoclastogenesis (15, 16). RANKL, located on osteoblasts (17), interacts with its physiological receptor RANK which is expressed as a type I membrane receptor on osteoclasts and their hematopoietic precursors of the monocyte/macrophage-lineage. Binding of RANKL to its receptor leads to increased differentiation of precursor cells, as well as activation of mature osteoclasts. The biological activity of RANKL is negatively regulated by the secreted decoy receptor osteoprotegerin that binds and sequesters RANKL. The regulation of osteoclast differentiation and activity by osteoblasts therefore couples osteoclastic bone resorption to osteoblastic bone formation and constitutes a local regulatory mechanism for bone remodeling.

Besides osteoblasts, cells of human dental tissues such as periodontal ligament cells have been found to be parathyroid hormone-responsive (18, 19) and also express components of the RANK/RANKL/osteoprotegerin system (20, 21). Periodontal ligament cells, together with collagenous and elastic fibers, form the periodontal ligament that connects the tooth root to the alveolar bone and is essential for maintaining the structural and functional integrity of the dentition. Periodontal ligament cells were demonstrated to exhibit *in vitro* phenotypic characteristics consistent with osteoblast-like cells (22), such as increased production of alkaline phosphatase in response to $1\alpha,25(OH)_2D_3$ stimulation and intense immunostaining for osteocalcin, suggesting that these cells have the potential to differentiate into osteoblasts (23). Furthermore, periodontal ligament cells were reported to respond to hormonal stimulation (24) as well as to tensile mechanical stress with changes in the osteoprotegerin/RANKL ratio (25). Several pieces of evidence indicate that, besides targeting bone, parathyroid hormone may also regulate the activities of periodontal tissues. Parathyroid hormone and parathyroid gland extract have been found to enhance tooth eruption and orthodontic tooth movement (26), tissues within the developing periodontal ligament to possess parathyroid hormone-binding sites (27) and certain strains of parathyroid hormone related protein-deficient mice to exhibit failure of tooth eruption and ankylosis of cementum with surrounding alveolar bone (28).

Whether the effect of an intermittent parathyroid hormone challenge of periodontal ligament cells on periodontal regeneration might at least partially be mediated by an altered production of osteoprotegerin and RANKL has not been investigated so far. Besides regenerative processes following inflammatory periodontal disease, this question is of particular interest with respect to support of the osteointegration of dental implants (29) and formation of reparative cementum occurring after tooth root resorption induced by orthodontic tooth movement.

Therefore, we addressed this question in the present investigation and hypothesized that an intermittent parathyroid hormone treatment would exert a dose- and time-dependent effect on the proliferation, differentiation and production of osteoprotegerin and RANKL in human periodontal ligament cells in a maturation-state dependent manner. We speculated that intermittent parathyroid hormone application would result in elevated levels of osteoprotegerin mRNA and protein as opposed to a decrease in RANKL expression and production resulting in a modulation of the osteoprotegerin/RANKL ratio favoring osteogenesis over bone resorption.

Materials and methods

Cell culture

Periodontal ligament cells were explanted from the bicuspids of six different human donors, aged between 12 and 14 years, showing no clinical signs of periodontitis. The teeth had been extracted for orthodontic reasons, with informed parental consent and following an approved protocol of the ethics committee of the University of Bonn. Periodontal tissue was scraped from the middle third of the roots to eliminate contamination of the culture with gingival fibroblasts or pulp cells. Fourth to eighth passage cells were plated at 9300 cells/cm^2 in 24-well plates ($n = 6$) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5000 U/ml penicillin and 5000 U/ml streptomycin; Biochrom AG, Berlin, Germany) and

cultured at 37°C in an atmosphere of 100% humidity and 5% CO_2. Ascorbic acid was not added to the media. The cells of different donors were used at the same passage. Media were changed at 24 h and then every 48 h until the cells reached confluence. Each experiment was performed with cells from all six donors and not only from a subset of donors. For experiments with preconfluent cells, cells were plated such that they remained in a subconfluent state at the end of culture (~90%) and were treated following a culture period of 24 h.

Commercially available human dermal fibroblasts (1BR.3.G; European Collection of Cell Cultures) and human MG63 osteoblast-like cells (European Collection of Cell Cultures) were cultured according to the manufacturer's instructions and served as reference cell lines for the characterization of the periodontal ligament cells, as well as for the comparison of periodontal ligament cell response to parathyroid hormone stimulation.

Characterization of the cells

RNA isolation, reverse transcription — Total RNA was isolated from all cell lines employed using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Afterwards, RNA was reverse transcribed with the respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alkaline phosphatase, osteocalcin, osteoprotegerin and RANKL antisense primer (Invitrogen, Carlsbad, CA, USA; for primer sequences see Table 1).

Polymerase chain reaction — The amplification was performed according to standard protocols including 29 cycles of denaturation, annealing and extension. The cycle number had been determined in other experiments using the same primers for real-time PCR to assure that the cycle number did not exceed the linear part of the amplification. The levels of amplified DNAs were visualized by running the PCR products in a 1.5% agarose gel and quantified using the AlphaDigiDoc1000 program by averaging three separate measurements and normalized to the endogenous reference gene GAPDH. Samples without reverse transcriptase treatment served as controls.

Immunofluorescence microscopy — In order to detect the marker proteins, cells on glass cover-slips were fixed and permeabilized using a commercially available kit (Fix & Perm, Bioresearch GmbH, Kaumberg, Austria) followed by incubation with the respective primary antibody in a 1:50 working solution of Tris-buffered saline/bovine serum albumin for 1 h at 37°C [monoclonal mouse anti-human alkaline phosphatase (Biomol GmbH, Hamburg, Germany), monoclonal mouse anti-human osteocalcin (Zymed, South San Francisco, CA, USA), polyclonal goat anti-human parathyroid hormone-receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA)]. Thereafter, cells were rinsed and incubated with a goat anti-mouse or rabbit anti-goat Cy3-conjugated immunoglobulin (Dianova GmbH, Hamburg, Germany) diluted 1:200 or 1:400 in Tris-buffered saline/bovine serum albumin for 1 h at room temperature. Following further rinsing and counterstaining with Hoechst 33258 (Sigma Aldrich, St Louis, MO, USA) to identify nuclei, specimens were cover-slipped for microscopical analysis.

In order to prove the specificity of the immunoreactions, negative controls were carried out by (i) omitting the primary antibody, (ii) omitting both the primary and secondary antibody and using Tris-buffered saline/bovine serum albumin instead and (iii) substituting an isotype matched mouse IgG-1 (Medac, Hamburg, Germany) for the primary antibody.

MG63 cells served as positive control for all antibodies used because they have been shown to express basal levels of the selected markers (30).

Parathyroid hormone treatment

Since dose-dependent and maturation state-dependent effects of an intermittent parathyroid hormone treatment have been reported in osteoblasts (31), preconfluent and confluent cells were cultured in the presence of low dose parathyroid hormone (1–34) (10^{-12} M) vs. high doses (10^{-8} M and 10^{-7} M) (Sigma Aldrich) for 0, 1, 3, 6 and 24 h within a 48 h incubation cycle. For the remaining time, experimental media were replaced by tissue culture media. These cycles were carried out three times. Since we did not observe any dose-dependence of the parathyroid hormone (1–34) effect in confluent periodontal ligament cells, we only employed 10^{-12} M parathyroid hormone

Table 1 Primer sequences used for reverse transcription–polymerase chain reaction

Gene	Sequence	Product size
GAPDH	(5′-3′): CGTCTTCACCACCATGGAGA (sense) (5′-3′): CGGCCATCACGCCACAGTTT (antisense)	300 bp
Alkaline phosphatase	(5′-3′): TGGAGCTTCAGAAGCTCAACACCA (sense) (5′-3′): ATCTCGTTGTCTGAGTACCAGTCC (antisense)	453 bp
Osteocalcin	(5′-3′): CATGAGAGCCCTCACA (sense) (5′-3′): AGAGCGACACCCTAGAC (antisense)	314 bp
Parathyroid hormone-receptor	(5′-3′): ACCAATGAGACTCGTGAACGG (sense) (5′-3′): AAGGACAGGAACAGGTGCATG (antisense)	166 bp
Osteoprotegerin	(5′-3′): GCTAACCTCACCTTCGAG (sense) (5′-3′): TGATTGGACCTGGTTACC (antisense)	324 bp
RANKL	(5′-3′): GCCAGTGGGAGATGTTAG (sense) (5′-3′): TTAGCTGCAAGTTTTCCC (antisense)	486 bp

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RANKL, receptor activator of nuclear factor kappaB ligand.

(1–34) in our experiments with preconfluent periodontal ligament cells.

Untreated cells and cells cultured in the presence of the parathyroid hormone-vehicle served as controls. Human dermal fibroblasts and MG63 osteoblast-like cells were used as a reference.

Cell proliferation

At harvest, cells were released from the culture surface by trypsinization for 10 min at 37°C. This reaction was terminated by the addition of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Thereafter, the cell suspension was centrifuged at 500 *g* for 10 min. The cell pellet was rinsed with phosphate-buffered saline and resuspended in 0.9% NaCl. Finally, cell number was determined by the use of a cell counter (Moelab, Hilgen, Germany). Cells harvested in this manner exhibited > 95% viability based on Trypan blue exclusion.

Parathyroid hormone effect on differentiation

Alkaline phosphatase specific activity was measured at the protein level in lysates of isolated cells as described previously (32). The level of osteocalcin in the conditioned media was assayed using a commercially available enzyme-linked immunoassay (ELISA) kit specific for human osteocalcin (IBL GmbH, Hamburg, Germany).

Parathyroid hormone effect on key regulatory factors of bone remodeling

Changes in levels of osteoprotegerin and RANKL were assessed both at the transcriptional level (as described above) and at the protein level using commercially available enzyme-linked immunoassay (ELISA) kits (Immundiagnostik AG, Bensheim, Germany). Changes in the production of osteoprotegerin and soluble RANKL (sRANKL) were assessed as a function of cell number and protein content (BCA protein assay kit, Pierce, Rockford, IL, USA).

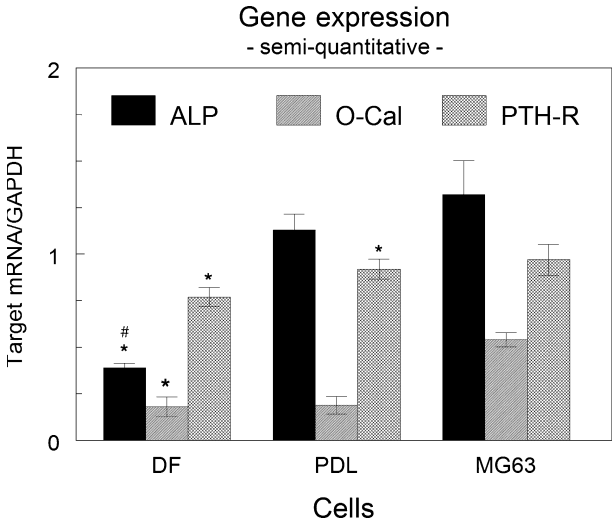

Fig. 1. Osteoblastic marker gene expression in periodontal ligament cells, dermal fibroblasts and MG63 osteoblast-like cells. Cells were cultured to confluence in 24-well plates. Following RNA isolation, semiquantitative reverse transcription–polymerase chain reaction was performed. Gene expression was assessed by averaging three separate measurements and normalized to the endogenous reference gene GAPDH. Each value is the mean ± SEM for six independent cultures. Data are from one of two separate determinations, which yielded comparable results. *$p < 0.05$, cell type vs. MG63 cells; $p < 0.05$, dermal fibroblasts vs. periodontal ligament cells. ALP, alkaline phosphatase; DF, dermal fibrobasts; GAPDH, glyceraldeyde-3-phosphate dehydrogenase; O-Cal, osteocalcin; PDL cells, periodontal ligament cells; PTH-R, parathyroid hormone-receptor.

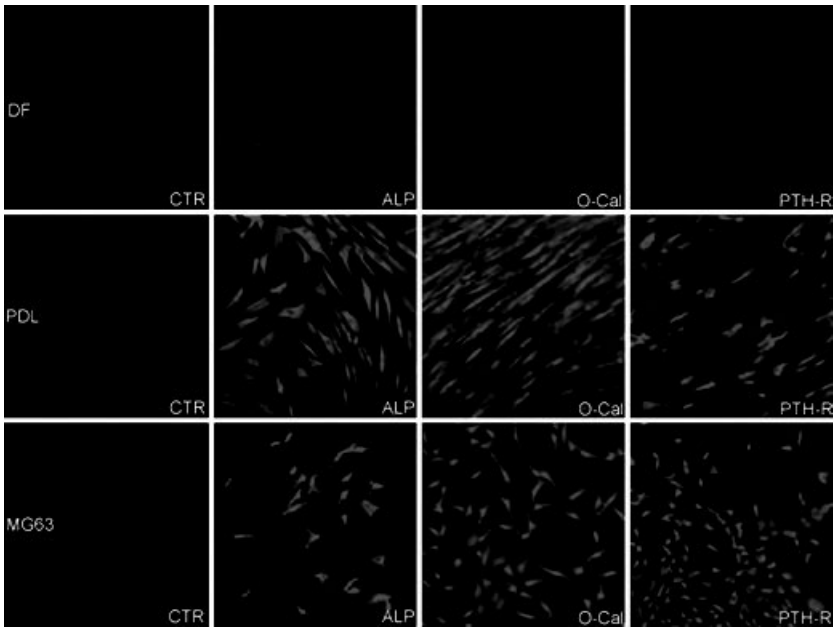

Fig. 2. Fluorescence immunohistochemical characterization of periodontal ligament cells (PDL), dermal fibroblasts (DF) and MG63 osteoblast-like cells. In confluent periodontal ligament and MG63 cells, cytoplasmic staining of granular type with various intensities from moderate to strong was observed for alkaline phosphatase activity (ALP), osteocalcin (O-Cal) and parathyroid hormone-receptor (PTH-R), whereas DF cells exhibited only weak immunoreactivity. No staining was observed in the negative control experiments (CTR).

Statistical analysis

For any given experiment, each data point represents the mean ± SEM of six independent cultures. Variance and statistical significance of data were analyzed using Bonferroni's modification of Student's *t*-test. *p*-values < 0.05 were considered to be significant. Each set of experiments was repeated twice and analyzed separately, and both sets of experiments had comparable results. Only results from one of the two sets of experiments are presented.

Results

Characterization of the cells

Compared to the osteoblastic reference cell line MG63, periodontal ligament cells expressed similar amounts of alkaline phosphatase and parathyroid hormone-receptor mRNA, whereas lower levels of osteocalcin mRNA were detected. These differences proved statistically significant ($p < 0.05$). As for dermal fibroblasts, ratios of the different marker genes to one another were similar to periodontal ligament and MG63 cells but the overall expression levels were significantly lower (Fig. 1).

At the protein level, cytoplasmic immunoreactivity was observed for alkaline phosphatase activity and osteocalcin in periodontal ligament cells. All immunoreactive cells showed granular type cytoplasmic staining with various intensities from moderate to strong. Periodontal ligament cells also showed staining for parathyroid hormone-receptor. In MG63 cells, all marker proteins were found to be expressed in about the same localization and intensities as in periodontal ligament cells, whereas in dermal fibroblasts, only weak immunoreactivity was observed for any of the markers evaluated. No immunostaining was observed in any of the negative control experiments (Fig. 2).

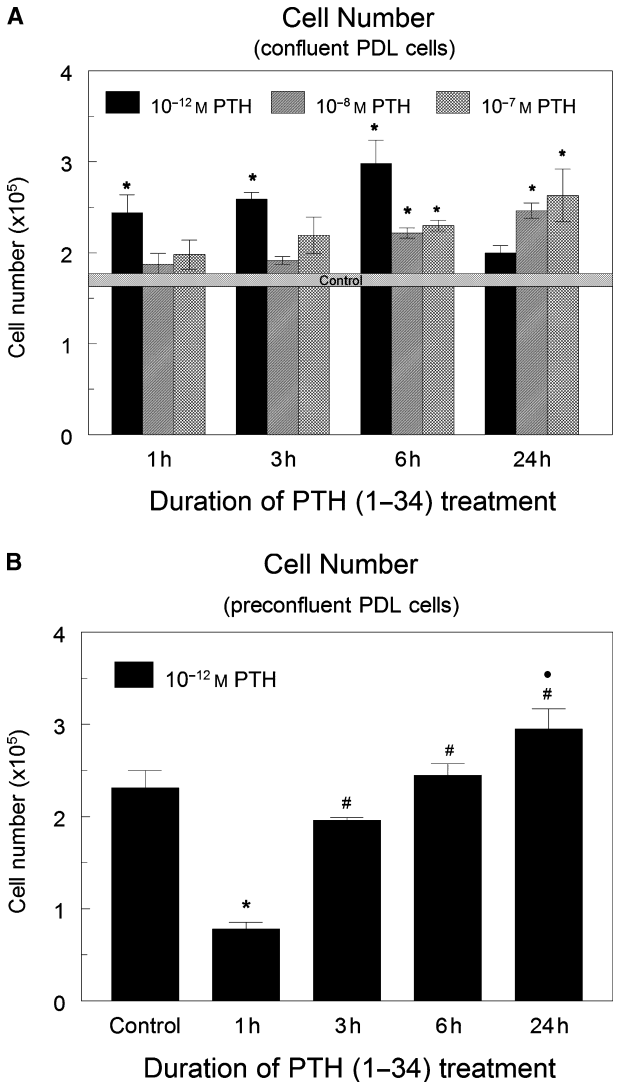

Fig. 3. Effect of an intermittent parathyroid hormone (1–34) treatment on regulation of cell number in confluent (A) and preconfluent periodontal ligament cells (B). Periodontal ligament cells were either treated with vehicle or 10^{-12} M, 10^{-8} M or 10^{-7} M parathyroid hormone (1–34) for 0, 1, 3, 6 or 24 h during three cycles of 48 h each. Data are from one of two separate experiments, which yielded comparable results. Each value is the mean ± SEM for six independent cultures. *$p < 0.05$, experimental group vs. untreated control; $p < 0.05$, experimental group vs. experimental group exposed to parathyroid hormone (1–34) for 1 h/cycle; •$p < 0.05$, experimental group vs. experimental group exposed to parathyroid hormone (1–34) for 1 h, 3 h/cycle. PDL cells, periodontal ligament cells; PTH, parathyroid hormone.

Effect of the intermittent parathyroid hormone (1–34) treatment on cell number

Intermittent parathyroid hormone (1–34) treatment caused a significant time- but not dose-dependent increase in cell number in confluent periodontal ligament cells, with a maximum increase of 32% following intermittent parathyroid hormone application at a concentration of 10^{-12} M for 6 h within each 48 h incubation cycle. A similar but less pronounced effect was observed for cultures exposed to higher doses of parathyroid hormone (10^{-8} M and 10^{-7} M), with cell numbers elevated by 31.8% following intermittent parathyroid hormone application at a concentration of 10^{-7} M for 24 h within each 48 h incubation cycle. Compared to the untreated controls, significantly higher cell numbers were observed after intermittent treatment for 6 h/cycle and 24 h/cycle ($p < 0.05$), whereas the increase in cell number following incubation of the cells in the presence of parathyroid hormone (1–34) for

1 h/cycle and 3 h/cycle displayed only a trend without statistical significance (Fig. 3A).

Similar results were obtained for the osteoblastic reference cell line MG63. All of the experimental groups displayed significantly higher cell numbers than the control cultures incubated in the absence of parathyroid hormone (1–34). In contrast, there was no effect of an intermittent parathyroid hormone (1–34) treatment in dermal fibroblasts with respect to proliferation (data not shown).

In preconfluent periodontal ligament cells, intermittent challenge with 10^{-12} M parathyroid hormone (1–34) led to a significant decrease of the cell number in response to a 1 h/cycle exposure. Longer parathyroid hormone treatments resulted in a gradual increase of cell numbers back to control levels (Fig. 3B). Again, similar observations were made for MG63 cells, whereas cell numbers were not affected in dermal fibroblasts (data not shown).

Effect of the intermittent parathyroid hormone (1–34) treatment on differentiation

In contrast to the increased cell numbers in confluent periodontal ligament cells caused by intermittent parathyroid hormone (1–34) challenge, there seemed to be a slight decrease in alkaline phosphatase activity in confluent periodontal ligament cells that only proved significant in the experimental groups exposed to 10^{-7} M parathyroid hormone (1–34) for 3 h/cycle and 6 h/cycle as opposed to an increase in preconfluent periodontal ligament cells after parathyroid hormone (1–34) challenge for 24 h/cycle (Figs 4A and B).

The same treatment regimen resulted in a gradual reduction in osteocalcin protein levels in confluent cells dependent on the exposure time per cycle (Fig. 5A), whereas in preconfluent periodontal ligament cells, differentiation was enhanced as shown by the significant increase in osteocalcin production following intermittent parathyroid hormone (1–34) exposure for 1 h/cycle. Longer presence of parathyroid hormone (1–34) in the cultures resulted in a decline to control levels (Fig. 5B).

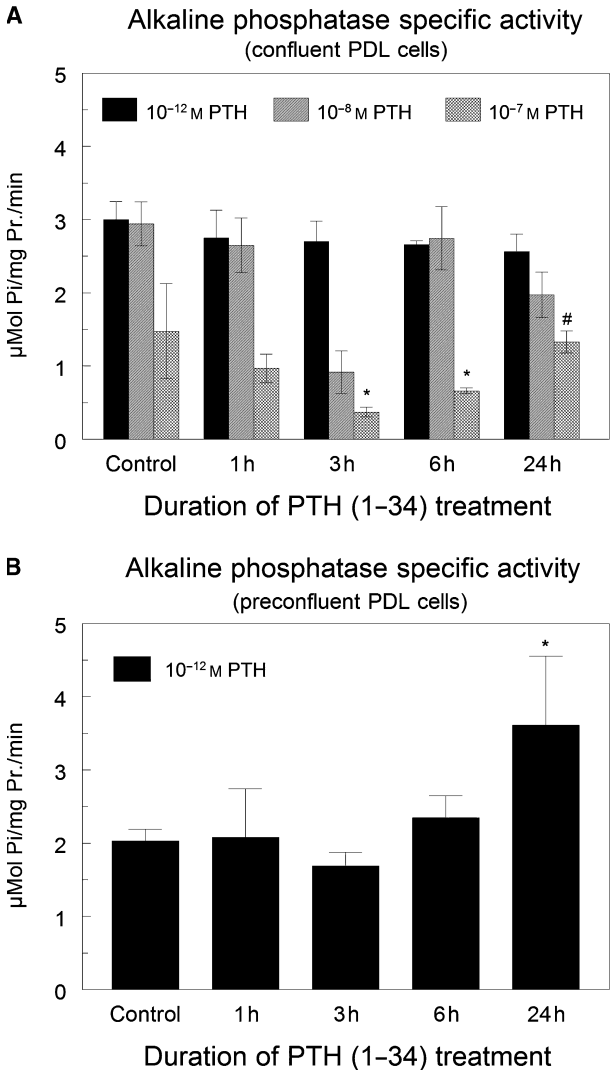

Fig. 4. Reduction in alkaline phosphatase specific activity in confluent periodontal ligament cells (A) as opposed to an increase following parathyroid hormone (1–34) administration for 24 h/cycle in preconfluent cells (B). Cells were treated with vehicle or 10^{-12} M, 10^{-8} M or 10^{-7} M parathyroid hormone (1–34) for 0, 1, 3, 6 or 24 h during three cycles of 48 h each. Data are from one of two separate experiments, which yielded comparable results. Each value is the mean ± SEM for six independent cultures. *$p < 0.05$, experimental group vs. untreated control; $p < 0.05$, experimental group vs. experimental group exposed to parathyroid hormone (1–34) for 3 h, 6 h/cycle. PDL cells, periodontal ligament cells; PTH, parathyroid hormone.

Similar results were again obtained for MG63 cells whereas osteocalcin was not detectable in dermal fibroblasts (data not shown).

Effect of the intermittent parathyroid hormone (1–34) treatment on expression and production of osteoprotegerin and soluble RANKL

Following incubation of confluent periodontal ligament cells with parathyroid hormone (1–34), osteoprotegerin mRNA expression decreased in all experimental groups. The mRNA levels were reduced compared to the untreated control cultures, with the least reduction after parathyroid hormone (1–34) treatment for 6 h/cycle (Fig. 6A). At the protein level, osteoprotegerin production was reduced dependent on the exposure time per cycle, with the most pronounced effect (−59.7%) in the experimental group treated with 10^{-7} M parathyroid hormone (1–34) for 24 h/cycle (Fig. 7A).

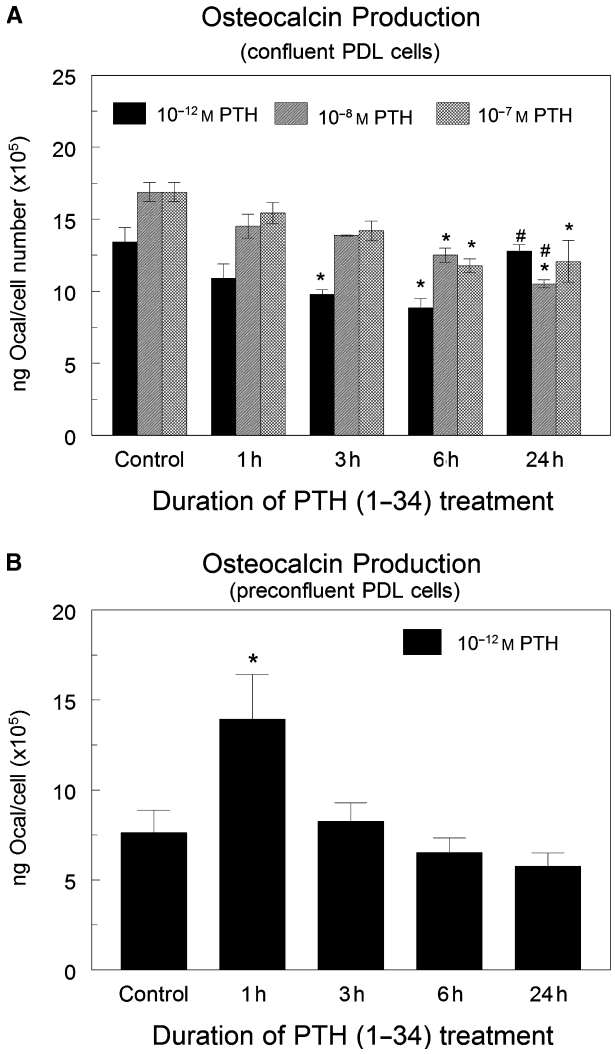

Fig. 5. Decrease in osteocalcin production in confluent periodontal ligament cells (A) following intermittent exposure to parathyroid hormone (1–34) as opposed to an increase in preconfluent periodontal ligament cells (B). Cells were treated with vehicle or 10^{-12} M, 10^{-8} M or 10^{-7} M parathyroid hormone (1–34) for 0, 1, 3, 6 or 24 h during three cycles of 48 h each. Data are from one of two separate experiments, which yielded comparable results. Each value is the mean ± SEM for six independent cultures. *$p < 0.05$, experimental group vs. untreated control; $p < 0.05$, experimental group vs. experimental group exposed to parathyroid hormone (1–34) for 1 h, 3 h, 6 h/cycle. Ocal, osteocalcin; PDL cells, periodontal ligament cells; PTH, parathyroid hormone.

In preconfluent periodontal ligament cells, osteoprotegerin mRNA and protein levels were not affected by intermittent parathyroid hormone (1–34) exposure except for the 6 h/cycle treatment group, where osteoprotegerin protein production was elevated by 49.2% compared to the untreated control (Figs 6B and 7B).

Soluble RANKL mRNA and sRANKL antigen could not be detected in our periodontal ligament cells. The results obtained for osteoprotegerin and sRANKL mRNA and protein in MG63 cells resembled the data for periodontal ligament cells.

Neither osteoprotegerin nor sRANKL was detectable in dermal fibroblasts, either at the transcriptional or at the protein level.

Discussion

In this study, the effect of an intermittent parathyroid hormone exposure of periodontal ligament cells in terms of proliferation, differentiation, and production of local factors important to bone remodeling was investigated with respect to the importance of the time-course and dose-dependency of the parathyroid hormone administration, as well as the maturation state of the cells employed.

We found periodontal ligament cells to express several osteoblastic markers, which is in line with previous reports in the literature (23). In the dermal fibroblast reference cell line, these markers could be demonstrated at the protein level but were only translated into proteins to a low degree, as shown by weak immunostaining. There are reports in the literature confirming the presence of alkaline phosphatase in skin fibroblasts (33), and parathyroid hormone-receptors have also been demonstrated in these cells (34), supporting the possibility that in our culture of dermal fibroblasts, there were several immunoreactive cells carrying small amounts of the antigen.

In order to examine the effect of an intermittent parathyroid hormone challenge of periodontal ligament cells on proliferation, the cell number was determined. It is evident that changes in cell number might result from altered proliferation and also from shortened or prolonged survival due to modulation of apoptosis. The latter has been evaluated in mesenchymal cells and MC3T3-E1 cells by Chen and coworkers (35). Their data suggest that parathyroid hormone accelerates turnover of osteoblasts by promoting cell viability early and promoting cell departure from the differentiation program later in their developmental scheme. Furthermore, apoptosis may be proliferation-independent or proliferation-dependent. Our assessment does not allow any conclusion as to whether parathyroid hormone exerts its effect on proliferation or apoptosis or both. Preliminary experiments in our laboratory looking at BrdU-incorporation into periodontal ligament cells intermittently exposed to parathyroid hormone have indicated that the increase in cell number observed in confluent periodontal ligament cells correlates with increased numbers of BrdU-positive cells

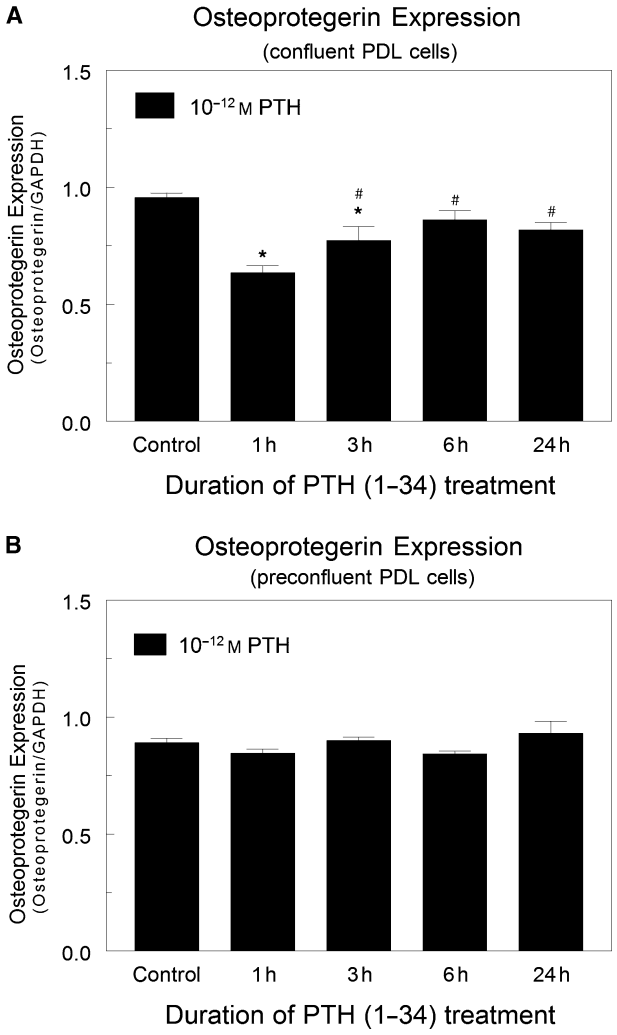

Fig. 6. Intermittent parathyroid hormone (1–34) treatment caused a reduction in osteoprotegerin mRNA expression in confluent periodontal ligament cells (A) as opposed to a time-dependent decrease of osteoprotegerin protein levels (B). Periodontal ligament cells were treated with vehicle or with 10^{-12} M, 10^{-8} M and 10^{-7} M parathyroid hormone (1–34) for 0, 1, 3, 6 or 24 h during three cycles of 48 h each. Data are from one of two separate experiments, which yielded comparable results. Each value is the mean ± SEM for six independent cultures. $*p < 0.05$, experimental group vs. untreated control; $p < 0.05$, experimental group vs. experimental group exposed to parathyroid hormone (1–34) for 1 h/cycle (A) and for 1 h, 3 h, 6 h/cycle, respectively (B). PDL cells, periodontal ligament cells; PTH, parathyroid hormone.

(unpublished data). This does not exclude a parathyroid hormone effect on apoptosis of periodontal ligament cells, of course.

As for the parathyroid hormone effect with respect to differentiation, Isogai *et al.* (31) pointed out the importance of cell density and differentiation-stage of the cells. In their model using primary osteoblast-like cells isolated from newborn mouse calvaria, parathyroid hormone treatment of subconfluent cells resulted in an increased alkaline phosphatase activity and osteocalcin production, whereas the same treatment regimen dose-dependently reduced alkaline phosphatase activity and osteocalcin levels in confluent cells. The authors concluded that parathyroid hormone may preferentially stimulate osteoblast differentiation in immature osteoblasts but inhibit it in more mature cells. Similar observations were made in our study with periodontal ligament cells. These findings give further support to the idea that although the mixed population of periodontal ligament cells mainly comprises fibroblasts, a certain percentage of these cells display traits typical of osteoblasts. This does not only hold true for marker gene expression but also for their response to hormonal stimulation. Apparently, there is a stronger parathyroid hormone influence on steps occurring later in the cascade of differentiation than there is on early differentiation, as outlined by the stronger effect of parathyroid hormone on osteocalcin levels than on alkaline phosphatase activity. In contrast, Ishizuya *et al.* (2) applied a similar treatment protocol as we did to preconfluent osteoblastic cells and observed a suppression of alkaline phosphatase activity and mRNA expression for osteocalcin following intermittent parathyroid hormone exposure for 1 h/cycle, whereas parathyroid hormone challenge for 6 h within each cycle stimulated osteoblast differentiation. Additionally, in their study the effects were more apparent at the postconfluent phase. These results suggest that the diverse action of parathyroid hormone induced by different exposure times occurs at various stages of osteoblast differentiation, but is more prominent in mature than immature osteoblasts. Furthermore, these findings may be related to parathyroid hormone-receptor expression levels, which were found to increase with time in culture by the same group. According to the results obtained in primary osteoblast cultures presented by Wang *et al.* (36), the enhancement of differentiation might be more important to the anabolic effect of transient parathyroid hormone administration than an increase in the population of progenitor cells. However, different cell culture models employing primary or transformed cell lines and different treatment protocols might be taken into account in order to explain diverse results following intermittent parathyroid hormone exposure of osteoblasts (37).

Intermittent parathyroid hormone treatment of periodontal ligament cells also modulated osteoprotegerin production in a maturation state-dependent

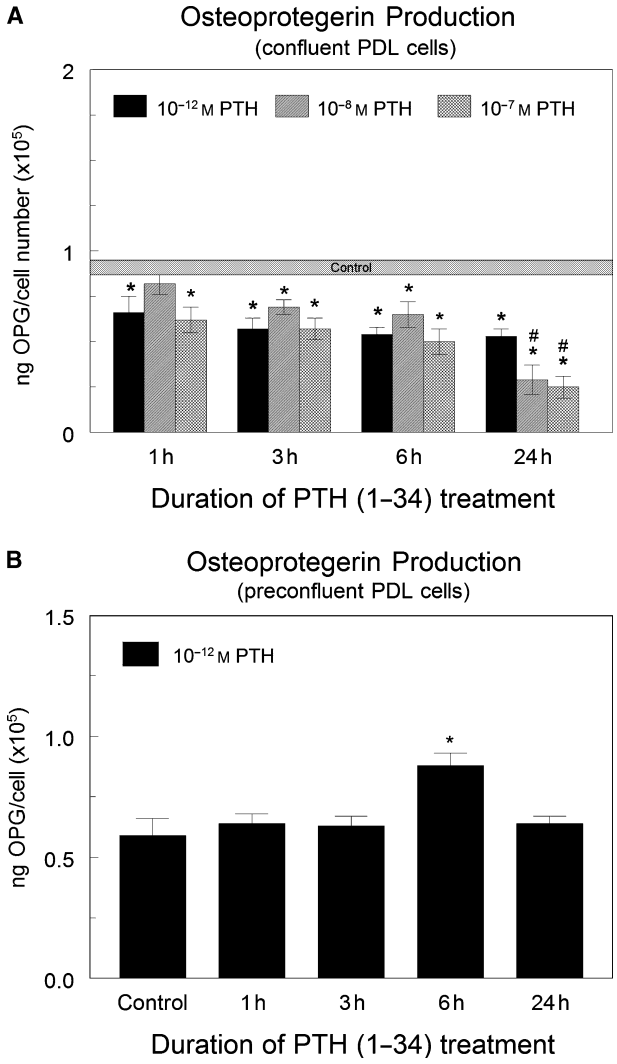

Fig. 7. Intermittent parathyroid hormone (1–34) treatment of preconfluent periodontal ligament cells does not alter osteoprotegerin mRNA expression significantly (A) but at the protein levels, there was a significant increase in osteoprotegerin production in response to an intermittent parathyroid hormone (1–34) challenge for 6 h/cycle (B). Periodontal ligament cells were cultured for 24 h prior to treatment with vehicle or with 10^{-12} M parathyroid hormone (1–34) for 0, 1, 3, 6 or 24 h during three cycles of 48 h each. Data are from one of two separate experiments, which yielded comparable results. Each value is the mean ± SEM for six independent cultures. *$p < 0.05$, experimental group vs. untreated control; $p < 0.05$, experimental group vs. experimental group exposed to parathyroid hormone (1–34) for 1 h, 3 h, 6 h/cyle. PDL cells, periodontal ligament cells; PTH, parathyroid hormone.

manner. Although there is an increasing bulk of literature showing that parathyroid hormone exerts its catabolic action through up-regulation of RANKL and inhibition of osteoprotegerin (38, 39), little is known about the influence of an intermittent parathyroid hormone challenge to these genes and how the state of differentiation of cells affects the responsiveness of these genes to parathyroid hormone. In a study with postmenopausal women treated with human parathyroid hormone (1–34), Buxton *et al.* (40) noted a rapid increase in serum levels of sRANKL but a mild suppression of osteoprotegerin within 6 months. Kostenuik *et al.* (41) found osteoprotegerin and parathyroid hormone to have additive effects on bone density and mechanical strength in osteopenic ovariectomized rats, but the influence of parathyroid hormone on osteoprotegerin levels was not investigated separately in this study.

The transient decrease in osteoprotegerin that we detected in mature periodontal ligament cells suggests that less osteoprotegerin protein would eventually be available at potential sites of osteoclast formation *in vivo*, whereas the significant increase in osteoprotegerin protein observed in preconfluent cells might result in favoring bone formation over bone resorption. Since osteoprotegerin suppresses the differentiation of osteoclasts, inhibits their activation, induces apoptosis (42) and counteracts the interaction of RANKL with its physiological binding partner RANK, the significant elevation of its level in preconfluent periodontal ligament cells and a possible increase in the osteoprotegerin/RANKL ratio plays a crucial role in coordinating the sequence of osteoclast differentiation during the bone remodeling cycle (43) by creating a microenvironment conducive to bone formation over bone resorption. However, sRANKL was not detected in our samples or in the control groups, nor was it produced in response to parathyroid hormone treatment. One possible interpretation is that sRANKL expression is not affected by these parameters. Alternatively, any changes that may have occurred were below the threshold of detection of the immunoassay kit we used. Support for the latter assumption comes from Granchi *et al.* (44), who observed basal expression of RANKL to be very low in MG63 osteoblast-like cells but to be inducible by the addition of interleukin-1β and various bone cement extracts to the cell culture medium in their study. This is in line with the increase in RANKL mRNA we observed in confluent MG63 cells in response to intermittent parathyroid hormone challenge. In contrast to the absence of RANKL mRNA and protein in our periodontal ligament control and experimental cultures, Kanzaki *et al.* (45) reported the expression of RANKL mRNA in periodontal ligament cells. Interindividual variations in specimens from different donors and different PCR protocols might be taken into account in order to explain this discrepancy. Finally, changes in osteoprotegerin transcription did not mirror the

changes at the protein level in our study, which is not surprising because alterations in transcription precede those in translation. Since gene expression and protein levels were measured after the same experimental period, it might very well be that we observed the changes in osteoprotegerin production, whereas osteoprotegerin mRNA levels had already returned to control levels.

In summary, our data shows that intermittent parathyroid hormone stimulation of periodontal ligament cells enhances the differentiation of preconfluent periodontal ligament cells and increases the release of local factors favouring bone formation over bone resorption whereas the opposite holds true for more mature periodontal ligament cells. In confluent periodontal ligament cells, intermittent parathyroid hormone exposure induces an inhibition of differentiation and osteoprotegerin production.

The data presented in this study provide evidence that periodontal ligament cells exhibit several osteoblastic traits and respond to intermittent parathyroid hormone administration with changes in osteoprotegerin production similarly to osteoblast-like cells and therefore bear the potential to play a key regulatory role in regenerative periodontal processes. Further experiments are needed in order to analyze the pathways and mediators involved and, more importantly, to see whether the altered osteoprotegerin levels we observed in response to parathyroid hormone administration are sufficient to actually result in a modification of the reparative properties of periodontal ligament cells or the resorptive activity of osteoclast-like cells.

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