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Aging affects the phenotypic characteristics of human periodontal ligament cells and the cellular response to hormonal stimulation *in vitro*

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Background and Objective: Aging modulates the proliferative activity and organic matrix production of cells *in vivo* and *in vitro*. Here, we explore how aging affects the phenotypic characteristics of human periodontal ligament cells and their response to hormonal stimulation.

Material and Methods: Fifth passage periodontal ligament cells from subjects aged 12–14 (group 1), 41–55 (group 2) and 61–70 years (group 3) were characterized for the expression of mesenchymal marker genes and proteins by real-time PCR and flow cytometry. Confluent cultures were exposed to 10^{-12} M parathyroid hormone(1–34) [PTH(1–34)] intermittently for three cycles. At harvest, cell number, alkaline phosphatase activity and osteocalcin production were determined by cell count, biochemical assay and ELISA.

Results: The characterization of the cells revealed a decreased expression of osteoblast-specific marker genes along with a lower percentage of cells presenting the respective proteins with age. An intermittent exposure of the cultures to 10^{-12} M PTH(1–34) induced an increase of the cell number as opposed to a significant decrease of alkaline phosphatase activity and osteocalcin production. The cellular response to PTH(1–34) was strongest in group 1. Basal osteoprotegerin levels were highest in the cultures from the oldest donors and inhibited by intermittent PTH(1–34) in all groups.

Conclusion: Our data indicate that periodontal ligament cells from older subjects display a less differentiated phenotype and a reduced response to intermittent PTH, suggesting a compromised ability to maintain tissue homeostasis and a limited possibility to support periodontal repair processes with age. The high basal osteoprotegerin expression in older subjects might serve as a compensatory mechanism.

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Several reports corroborate the observation that orthodontic tooth movement results in remodeling processes of the periodontal supportive apparatus (1–4). These remodeling processes seem to occur to a greater extent in adult individuals compared with juvenile patients and more frequently result in a greater loss of alveolar bone height in adults (5,6). The underlying differences of the biological reaction to the mechanical stimulus induced by the tooth movement have not yet been completely elucidated. In the light of an increasing number of adults seeking orthodontic treatment, a contribution to a better understanding of the role of cellular age for the regulation of the remodeling and repair processes underlying tooth movement would be desirable. Current knowledge regarding the role of the parameter 'age' is primarily based on *in vitro* investigations in various cell culture systems, e.g. epithelial cells, keratinocytes, lymphocytes and osteoblasts (7,8).

For the examination of the role of cellular age, two main approaches exist in the literature. On the one hand, cultures are subjected to an *in vitro* aging by repetitive cell passaging or, on the other hand, cells obtained from older donors are compared with cultures from juvenile donors.

Besides an age-dependent reduction of cellular function, including a reduced synthesis of DNA and extracellular matrix proteins (9,10), a further feature typical of *in vitro* aging of cells is a limited response to a stimulation with hormones and growth factors, leading to an interference with tissue homeostasis (7,8,11). For example, calcitriol was reported to induce an enhanced alkaline phosphatase specific activity in young cultures, whereas the effect faded with increasing cellular age (12,13). Likewise, osteoblasts and periodontal ligament cells display an inhibition of alkaline phosphatase specific activity with age (14). Furthermore, older periodontal ligament cells respond to specific stimuli with a more pronounced induction of inflammatory genes, such as the genes for prostaglandin E_2 (PGE_2), cyclooxygenase-2 and interleukin-1β, compared with cultures from younger donors (15–18).

In our previous investigations, different stages of confluence were employed as a model to reflect different states of cellular maturation. Preconfluent and confluent cultures displayed distinct expression patterns of differentiation parameters as well as distinct characteristics in the cellular response to a potentially anabolic intermittent administration of parathyroid hormone (1–34) [PTH(1–34)] (19–21). Besides these data, it remains unclear how the biological age affects PTH(1–34)-induced changes in the phenotypic expression of periodontal ligament cells and the production of local factors that regulate hard tissue remodeling. Addressing this question would widen the basic knowledge of the phenotypic characteristics and reparative potential of periodontal ligament cells and hold out the prospect of developing purposeful treatment strategies to interfere with alveolar bone loss and to support periodontal repair processes.

Therefore, it was the aim of the present investigation to compare the age-related expression levels of various differentiation parameters as well as of local factors involved in the regulation of alveolar bone metabolism in periodontal ligament cell cultures obtained from patients of different chronological age. In a second step, we aimed to compare the cellular response to stimulation with PTH(1–34) amongst those different age groups.

Material and methods

Periodontal ligament cell culture and cell characterization

Human periodontal ligament cells were scraped from the middle third of the roots of premolars of five donors per age group. Age groups were defined as follows: group 1, 12–14 years old; group 2, 20–40 years old; and group 3, 60–75 years old. The donors did not show clinical signs of periodontitis, nor did they suffer from any systemic disease that would interfere with the periodontal health or require anti-inflammatory or antimicrobial therapy. The teeth had been extracted for orthodontic or prosthodontic reasons, with written informed consent and following an approved protocol of the ethics committee of the University of Bonn (reference number 029/08).

Prior to PTH(1–34) stimulation, fifth passage cells of each age group were characterized for the senescence-related expression of caveolin-1 and several markers (total of 84) that are typical of mesenchymal cells by means of microarray (SABiosciences, Frederick, MD, USA) including *alkaline phosphatase (ALP), osteocalcin, bone morphogenetic protein (BMP)-2, BMP-4, BMP-7, integrin α6, integrin β4, transforming growth factor*-β, *Notch-1, connexin-43*, and *growth/differentiation factor-5*. Striking differences in gene expression were further quantified by real-time PCR. For characterization at the protein level, flow cytometry was carried out as described below.

Quantitative real-time PCR— Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Afterwards, 1 μg of RNA was reverse transcribed with 200 ng of random primers in a 15 μL volume using the Amersham-Pharmacia-Biotech RT kit (Amersham Biosciences, Piscataway, NJ, USA). Optimal oligonucleotide primers for the analysis of *caveolin-1, PTH receptor, ALP, osteocalcin, BMP-2, BMP-4, BMP-7, integrin α6, integrin β4, osteoprotegerin (OPG)* and *RANKL* mRNA expression were purchased from Qiagen, Hilden, Germany. Real-time PCR was performed on a light-cycler (Roche, Mannheim, Germany) using the light cycler software version 3.5.3. The PCRs were carried out in a total volume of 20 μL in PCR master mix containing 10 μL SYBR® green, 2 μL of 10× QuantiTect primer assay and 2 μL of the reverse transcription product made up to 20 μL with RNase-free H_2O. The amplifications were performed in duplicate for each sample, and the optimal annealing temperature for all primers was 55°C for 40 cycles. To normalize the content of cDNA samples, the comparative threshold (Ct) cycle method was used, comprising normalization of the number of target gene copies vs. an endogenous reference gene, such as *GAPDH*. For comparative analysis of

gene expression, the $\Delta\Delta$Ct method was applied. Through this method, a single sample, represented in our experiments by cells without any treatment, was designed as a calibrator and used for comparison of gene expression level of any unknown samples.

Flow cytometry analysis— For further characterization, flow cytometry was performed to identify and quantify the subpopulation of periodontal ligament cells that express caveolin-1, ALP and osteocalcin protein. Briefly, cells were released from the surface of the culture wells by the use of accutase®, and fixed and permeabilized (Fix & Perm kit; BD Biosciences, Heidelberg, Germany) to allow for a binding of the primary antibodies to the antigens that are expressed in the cytoplasm. Following incubation with the respective primary antibodies (for caveolin-1, rabbit anti-human, Santa Cruz, Heidelberg, Germany; for ALP, rabbit anti-human, Quartett, Berlin, Germany; and for osteocalcin, mouse anti-human, Zytomed, Berlin, Germany) at a 1:50 dilution for 4 h at 37°C, the cells were rinsed, centrifuged and resuspended. Afterwards, the cells were incubated with the respective fluorescein isothiocyanate-labeled secondary antibody (anti-mouse, anti-rabbit; Santa Cruz) at a 1:400 working dilution for 1 h at room temperature in the dark. After another rinse, centrifugation and resuspension, the proportion of immunoreactive cells was quantified using the BD LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the BD FACSDiva software. Negative controls were carried out by using isotype-matched nonimmune IgGs instead of the primary antibodies.

Treatment with PTH(1–34)

Following characterization, fifth passage cells were plated in 24-well plates (10,000 per well; $n = 6$) and cultured to confluence 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) at 37°C in an atmosphere of 100% humidity and 5% CO_2 in air. Cells were not pooled. Confluent cells were cultured in the presence of 10^{-12} M PTH(1–34) (Sigma Aldrich, Seelze, Germany) for 1 and 24 h within a 48 h incubation cycle. For the remaining time, experimental media were replaced by tissue culture media without PTH(1–34). These cycles were carried out three times, resulting in a total experimental period of 6 d. This intermittent treatment regimen was used to mimic the potentially anabolic effects of PTH. Additionally, cells were subjected to continuous PTH(1–34) challenge in order to investigate whether the mode of PTH administration affects the cellular response. In these cultures, the experimental media were replaced every 2 d to assure that bioactive PTH (1–34) was continuously present in the culture system. Vehicle-treated cultures for each treatment group served as controls.

The effect of such a PTH(1–34) treatment regimen on the proliferative capacity of periodontal ligament cells, on the expression of differentiation parameters that are typical of osteoblasts and on the expression of genes that regulate hard tissue remodeling was assessed at the mRNA level by means of real-time PCR for ALP, osteocalcin, OPG and RANKL as described above.

At the protein level, alkaline phosphatase specific activity was measured in lysates of isolated cells as a function of release of *p*-nitrophenol from *p*-nitrophenylphosphate, as described previously (22). The levels of osteocalcin and osteoprotegerin protein in the conditioned media were assayed using commercially available ELISA kits specific for human osteocalcin and osteoprotegerin, respectively (IBL GmbH, Hamburg, Germany). Alkaline phosphatase, osteocalcin and osteoprotegerin protein were expressed as a function of the number of cells.

The cell number was determined by releasing the cells 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 and the cell pellet resuspended in 0.9% NaCl. Finally, the number of cells 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.

Calculation of results and statistical analysis

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

Results

Characterization

The transcript expression for the senescence-associated gene *caveolin-1* as well *ALP* and *PTH receptor* mRNA increased markedly with age, whereas the other osteoblastic marker genes decreased with age (Fig. 1A).

Flow cytometry analysis of the cells revealed a reduced number of cells that were immunopositive for caveolin-1 within age group 3 (92.31 ± 2.36% in age group 1; 91.13 ± 2.24% in age group 2; and 64.78 ± 3.75% in age group 3) as opposed to an increased percentage of cells carrying the osteoblastic marker protein alkaline phosphatase. The percentage of cells staining positively for osteocalcin was reduced with age (Fig. 1B). Serving as an example, Fig. 1C illustrates the fluorescence characteristics of a cell suspension from age group 1 for caveolin-1 and for the isotype-matched IgG control.

Effect of PTH(1–34)

The cell numbers were highest in the youngest age group and decreased

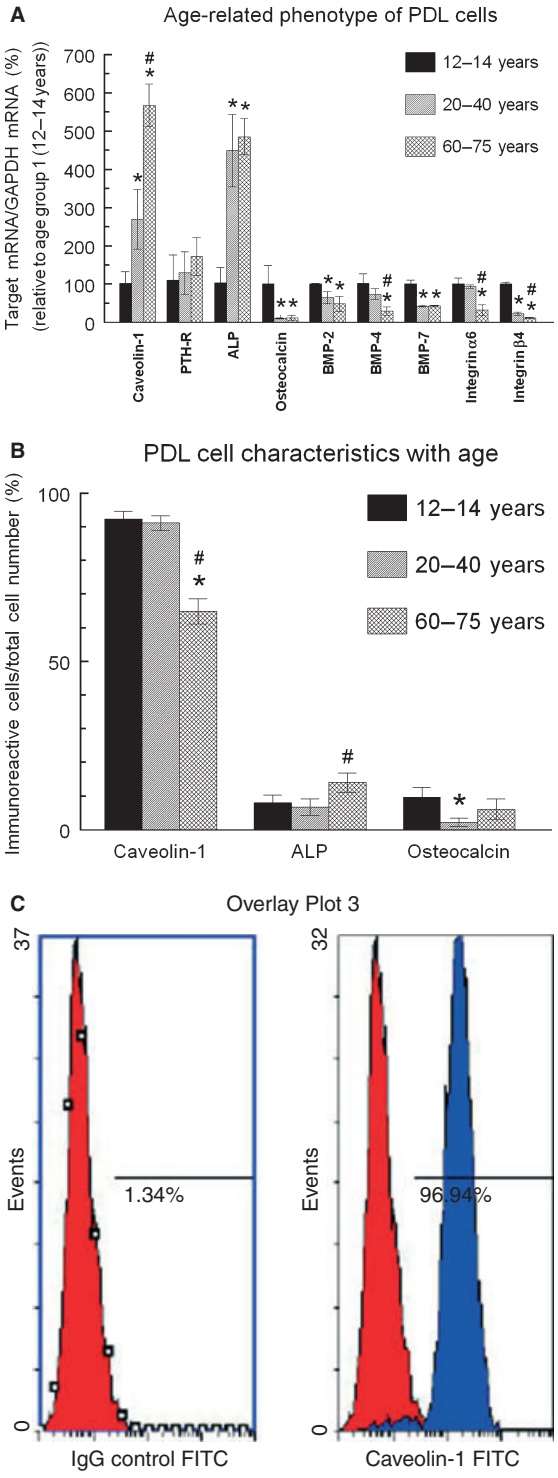

Fig. 1. Phenotypic characterization of fifth passage confluent human periodontal ligament cells by real-time PCR using cDNA of five different donors per age group (A) and by flow cytometry (B). For comparison, the gene expression in (A) is given as the relative expression compared with age group 1 (12–14 years), which served as a reference for normalization. Data are from two repetitive experiments, both yielding comparable results. $^{*}p < 0.05$, particular age group vs. age group 1 (12–14 years); $^{\#}p < 0.05$, age group 3 (60–75 years) vs. age group 2 (20–40 years). (C) Serving as an example, an overlay histogram measuring the fluorescence characteristics for caveolin-1 of a cell suspension from age group 1 and an isotype-matched control of the same age group is depicted.

with age. Intermittent treatment with PTH(1–34) caused a significant increase of the cell number in all age groups, with the most pronounced effect observed following PTH(1–34) for 24 h per cycle. The intensity of the cellular response to PTH(1–34) was strongest in the cultures of donors aged 60–75 years. A continuous administration of the hormone did not alter the cell numbers significantly in age groups 1 and 2, but caused an increase in age group 3 (Fig. 2).

Alkaline phosphatase specific activity decreased with age as evidenced by lower basal ALP levels in group 3 compared with group 2 compared with group 1. Intermittent exposure of the cultures to PTH(1–34) caused a significant decrease of ALP activity, with the most pronounced response to the hormonal stimulation seen in age group 1 following pulsatile PTH(1–34) for 24 h per cycle (Fig. 3).

Likewise, the results for osteocalcin resembled those obtained for ALP. The basal osteocalcin production was highest in cultures from younger donors and it decreased with age. When the cells were exposed to intermittent PTH(1–34), osteocalcin production was inhibited in all experimental groups, and the cellular response was most pronounced in age group 1 (Fig. 4). Induction of ALP and osteocalcin mRNA expression by PTH(1–34) did not correlate with the changes reported at the protein level but remained at subsignificant levels (data not shown).

Osteoprotegerin was expressed by cultures of all age groups. Levels of OPG were highest in age group 3, but those cultures showed the most distinct OPG reduction in response to an intermittent PTH(1–34) exposure compared with the cells of younger donors (Fig. 5). At the transcriptional level, the OPG/RANKL ratio was not modified significantly by this treatment regimen in age groups 1 and 2. However, intermittent PTH(1–34) induced a reduction of the basally highest ratio in the cultures of age group 3 (Fig. 6).

Discussion

In the light of the predicted increase of the number of people becoming older

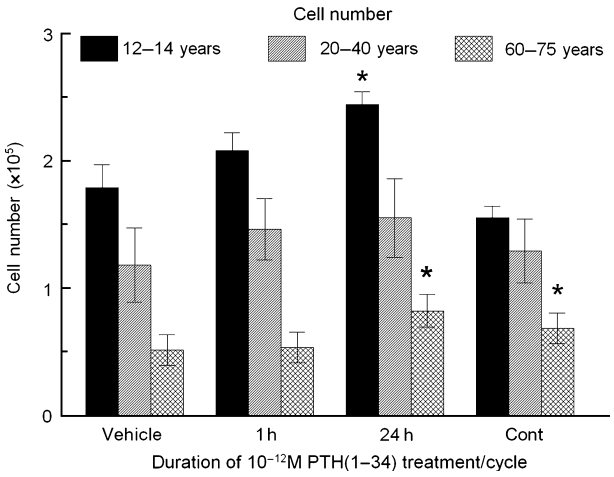

Fig. 2. Stimulatory effect of an intermittent 10^{-12} M PTH(1–34) administration on the regulation of the cell number in fifth passage confluent human periodontal ligament cells. Cells were treated intermittently with 10^{-12} M PTH(1–34) for 1 or 24 h during three cycles of 48 h each or challenged continuously (Cont). Ethanol-treated cultures served as vehicle controls (Vehicle). Since control groups did not differ significantly from each other, only one control per age group is presented. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for six independent cultures. $*p < 0.05$, experimental group vs. control within a particular age group.

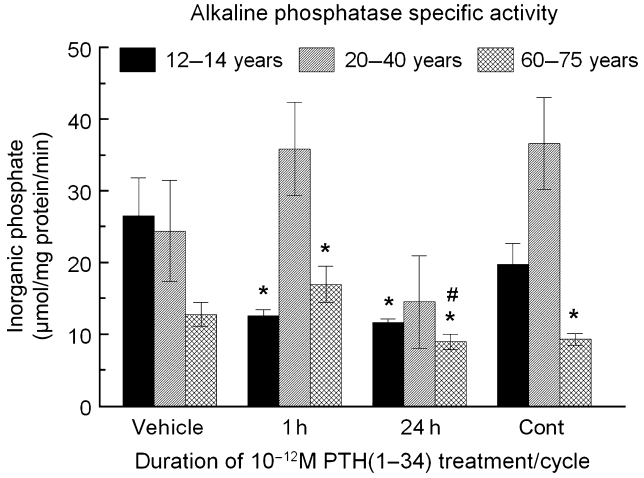

Fig. 3. Inhibition of alkaline phosphatase specific activity by parathyroid hormone (1–34) [PTH(1–34)] in human periodontal ligament cells. The relative effect was most pronounced in cultures from young donors (group 1). Cells were exposed to 10^{-12} M PTH(1–34) intermittently during three cycles or challenged continuously (Cont). Ethanol-treated cultures served as vehicle controls (Vehicle). The experiments were conducted twice, and both sets of experiments yielded comparable results. Each value represents the mean ± SEM for six independent cultures. $*p < 0.05$, experimental group vs. control within a particular age group. $^{\#}p < 0.05$, PTH(1–34) for 24 h per cycle vs. PTH(1–34) for 1 h per cycle within a particular age group.

than 65 years in the next decades (23), there is a clear demand for a better understanding of the cellular specificities of aged cells and of the age-related changes in the cellular response to pharmaceutical therapies, such as regenerative treatment approaches following periodontal tissue destruction.

The comparative characterization of human periodontal ligament cells of juvenile vs. aged donors in the present study revealed a significantly lower expression of osteoblastic marker genes in aged cultures, indicating a less differentiated osteoblastic phenotype and a compromised ability to maintain tissue homeostasis. The latter interpretation is further supported by the reduced expression of *integrin* α*6* and *integrin* β*4* mRNA in aged cultures, which suggests a limited ability of the cells to interact with neighbouring cells and the extracellular matrix (Fig. 1A). Along these lines, our flow cytometry data demonstrated an age-dependent increase of the percentage of cells that stained immunopositively for alkaline phosphatase (as a marker of early osteoblastic differentiation) as opposed to a reduced proportion of cells carrying the osteocalcin protein (as a marker of late osteoblastic maturation), also pointing towards a shift of the heterogeneous composure of periodontal ligament cells, with a lower proportion of cells exhibiting phenotypic traits of mature osteoblasts during the aging process (Fig. 1B). In support of this and mirroring the data of the present study, *in vitro* experiments in human periodontal ligament cells showed an age-dependent reduction of alkaline phosphatase specific activity (14). One might speculate that the apparent discrepancy between the decrease in ALP activity (Fig. 3) and the increase in *ALP* mRNA with age (Fig. 1A) observed in our experiments might be due to post-transcriptional regulatory mechanisms which cannot be presented at this point. The impression of a cellular dedifferentiation with age was corroborated by Lallier and coworkers (24), who reported a decrease of osteoblastic marker gene expression following sequential passaging of periodontal ligament cells in comparison to freshly isolated tissue.

The higher basal levels of osteoprotegerin we observed in aged periodontal ligament cell cultures (Fig. 5) might compensate for the limited osteoblastic function as part of a feedback mechanism. These findings are corroborated by the results of Benatti *et al.* (18), who concluded that aged periodontal ligament cells express an antiresorptive profile of enhanced *OPG* mRNA expression along with unaltered *RANKL* transcription in cultures from

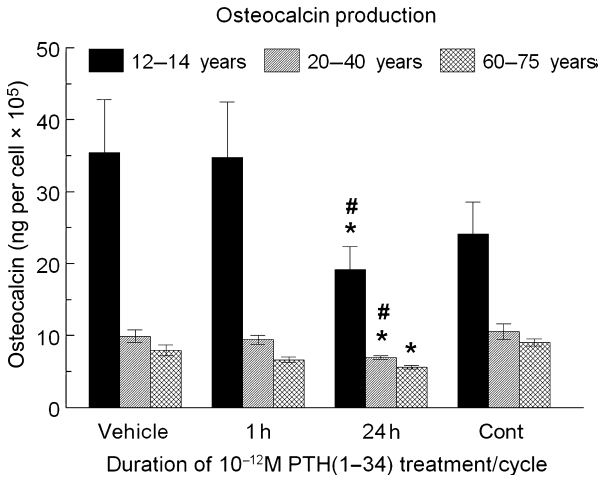

Fig. 4. Intermittent 10^{-12} M PTH(1–34) administration down-regulated the osteocalcin production by periodontal ligament cells significantly, and the strongest effect was observed in cultures from juvenile donors. Cells were treated intermittently with 10^{-12} M PTH(1–34) for 1 or 24 h during three cycles of 48 h each or challenged continuously (Cont). Ethanol-treated cultures served as vehicle controls (Vehicle). Data are representative of two independent experiments, both yielding comparable results. Each value is the mean ± SEM for six independent cultures. $^{*}p < 0.05$, experimental group vs. control within a particular age group; $^{\#}p < 0.05$, PTH(1–34) for 24 h per cycle vs. PTH(1–34) for 1 h per cycle within a particular age group.

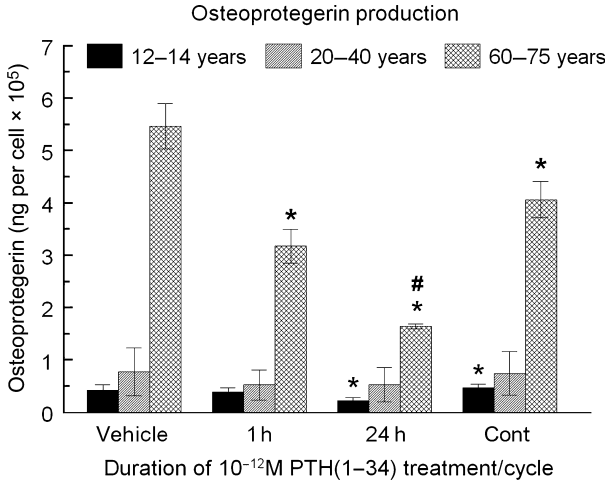

Fig. 5. The osteoprotegerin levels were highest in aged cultures in basal conditions but so was the PTH(1–34)-induced inhibition of its production. The cultures were challenged intermittently with 10^{-12} M PTH(1–34) for 1 or 24 h during three cycles of 48 h each or challenged continuously (Cont). Ethanol-treated cultures served as vehicle controls (Vehicle). Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for six independent cultures. $^{*}p < 0.05$, experimental group vs. control within a particular age group; $^{\#}p < 0.05$, PTH(1–34) for 24 h per cycle vs. PTH(1–34) for 1 h per cycle within a particular age group.

aged individuals. In accordance with this report, serum levels of OPG were found to be elevated in systemically healthy aged people, supporting the assumption that such an increase of OPG might serve the attempt to counteract the loss of bone mass associated with senescence (25).

Parathyroid hormone(1–34) modified the proliferation and differentiation parameters in this study, with aged individuals showing the most intense relative response with respect to the cell number (Fig. 2) and OPG levels (Fig. 5) but the least pronounced relative changes in alkaline phosphatase activity and osteocalcin production (Figs 3 and 4). Thus, the relative cellular response is parameter specific, and the extent to which those parameters can be modified by PTH is a function of age, but the data clearly demonstrate that aged periodontal ligament cells retain their ability to respond robustly to hormonal stimulation. However, the mechanism by which the intermittent PTH stimulation is translated into an anabolic cellular response in confluent aged cultures does not seem to be a direct induction of a more differentiated osteoblastic phenotype of periodontal ligament cells but rather an increase of the number of periodontal ligament cells, which might then be further directed by other factors in the local microenvironment. This conclusion is derived from the increase of the periodontal ligament cell number in response to intermittent stimulation with PTH (Fig. 2) as opposed to the inhibition of alkaline phosphatase activity and osteocalcin production by the same treatment regimen (Figs 3 and 4). In support to this notion, Friedl *et al.* (26) showed that in young, fast-growing rats, PTH induced an enhanced osteoblast activity, in contrast to an increased number of osteoblasts in aged rats.

A number of reports using the number of cells as the main measure indicate that the relative response to PTH increases with age (26,27) which supports our proliferation data (Fig. 2). However, these findings contradict our observations regarding the effect of PTH on periodontal ligament cell function, which was most pronounced in cultures from younger donors (Figs 3 and 4). It was suggested that the variation in the intensity of the effect of PTH amongst the different age groups might be related to a distinct expression density and activity of the PTH receptor (28–30). Real-time PCR analysis of our samples revealed an increased *PTH receptor* mRNA expression in specimens from aged donors, which explains the more intense effect

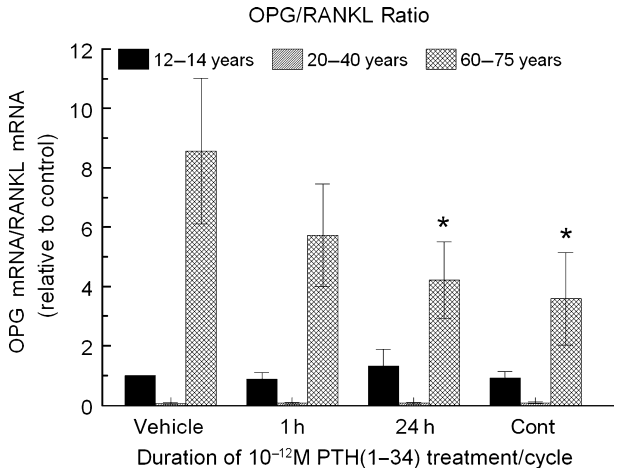

Fig. 6. Effect of PTH(1–34) on the OPG/RANKL ratio at the transcriptional level as determined by real-time PCR. The cultures were challenged intermittently with 10^{-12} M PTH(1–34) for 1 or 24 h during three cycles of 48 h each or challenged continuously (Cont). Ethanol-treated cultures served as vehicle controls (Vehicle). Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for six independent cultures. $*p < 0.05$, experimental group vs. control within a particular age group.

of PTH on cell number and osteoprotegerin production but not the limited response regarding the differentiation parameters.

The PTH-induced effects we observed at the protein level were not paralleled by similar changes at the transcriptional level, which mostly remained at a subsignificant level. This might have resulted from the time course of the PTH effect, which was demonstrated to exhibit a peak response within the first couple of hours upon exposure and to decline to control levels within 24 h (31–33). In our experiments, RNA was harvested after three PTH administration cycles of 48 h each (6 d total).

Our treatment protocols addressing the question of whether the mode of PTH administration affects the cellular response did not show any obvious converse effects of an intermittent vs. a continuous exposure to the hormone (Figs 2–5), but a lack of effect of continuous PTH administration for 6 d in all age groups. This observation might result from a down-regulation of the number or sensitivity of PTH receptors as part of a feedback loop. Unpublished data (S. Lossdörfer, D. Kraus, N. Abuduwali, A. Jäger) from our laboratory support the proposed mechanism. In these experiments, a long-term stimulation of periodontal ligament cultures with PTH(1–34) resulted in desensitization to a subsequent redosage.

Caveolin-1 was used as a senescence-associated marker in this study. The discrepancy between the data obtained at the transcriptional level (showing an increased expression with age; Fig. 1A) and the protein data (demonstrating less immunopositive cells with age; Fig. 1B) might be explained by the consideration that flow cytometry analysis only discriminates between immunoreactive and nonreactive cells but does not allow for a quantification of the intensity of the fluorescence signal of the single cell. Therefore, it might very well be that, in cultures from older donors, a smaller number of periodontal ligament cells express more caveolin-1 mRNA than the cells from younger donors.

In summary, the present investigation demonstrated that aging influences the phenotypic expression of human periodontal ligament cells and modifies the relative intensity of the cellular response to PTH(1–34) in a parameter-specific manner. These findings might explain the compromised ability of aged periodontal ligament cells to contribute to the maintenance of periodontal tissue homeostasis and limit the extent to which regenerative periodontal processes can be supported by PTH in aged individuals.

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