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Parathyroid hormone modifies human periodontal ligament cell proliferation and survival *in vitro*

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Background and Objective: Periodontal ligament (PDL) cells show traits that are typical of osteoblasts, such as osteoblastic marker gene expression and the ability to respond to parathyroid hormone (PTH) stimulation in an osteoblast-like manner with respect to differentiation and local factor production. In the present study, we hypothesized that human PDL cells might respond to PTH stimulation with changes in proliferation and cell survival and thereby provide another mechanism by which PTH might affect the reparative potential of PDL cells. We speculated that the maturation state of the cells and the mode of PTH(1-34) administration would have an impact on the cellular response.

Material and Methods: PDL cells were challenged with PTH(1-34) intermittently or continuously at different maturation states. Cell number, 5-bromo-2-deoxyuridine (BrdU) incorporation, DNA fragmentation, nitric oxide production and the duration of the PTH(1-34) effect were determined.

Results: Intermittent PTH(1-34) treatment of preconfluent cells caused a significant increase in proliferation and DNA fragmentation, whereas in more mature cells, proliferation was less enhanced while apoptosis was more pronounced than in immature cells. Continuous PTH(1-34) exposure did not alter proliferation in any maturation state but increased DNA fragmentation in preconfluent cells. PTH(1-34) prevented etoposide-induced apoptosis after 6 h but no longer after 24 h. Nitric oxide production was unaffected.

Conclusion: These results indicate that human PDL cells respond to PTH(1-34) with changes in proliferative and apoptotic signaling in a maturation-state-dependent manner. Besides changes in local factor production, these findings provide a further possible mechanism to support the idea that PDL cells possess the potential to be involved in the regulation of dental hard tissue repair.

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In several *in vitro* and *in vivo* model systems, it has been demonstrated that parathyroid hormone (PTH) is capable of exerting anabolic effects on bone when administered intermittently (1,2). At the cellular level, increased numbers of osteoblasts have been observed following the intermittent administration of PTH(1-34) and this was attributed, in part, to the stimulation of proliferation of osteoblast precursor cells as well as to the prolonged survival of mature osteoblasts owing to an anti-apoptotic effect. The latter is thought to be the predominant mechanism, as supported by an increasing mass of literature pointing at prolonged cell survival via inhibition of apoptosis in newly differentiated osteoprogenitor cells, as suggested by Jilka *et al.* (3). The reported effects of PTH on apop-

tosis appear to be dependent on the cell culture model (4,5) as well as on the differentiation state of the cells (6). PTH was found to be anti-apoptotic in preconfluent osteoblastic cells as opposed to pro-apoptotic in more differentiated postconfluent cells.

In addition to osteoblasts, periodontal ligament (PDL) cells were also reported to be PTH responsive (7–10). In previous research, we demonstrated that PTH(1-34) modulates the response of human PDL cells with respect to cell number, differentiation and production of key molecules of bone remodeling (9,10) in a maturation-state dependent manner. In these studies, intermittent PTH stimulation enhanced the differentiation of preconfluent PDL cells and increased the release of osteoprotegerin, favoring bone formation over bone resorption, whereas the opposite held true for more mature PDL cells. In confluent PDL cells, intermittent PTH exposure induced an inhibition of differentiation and osteoprotegerin production. These findings are of particular interest because they provide further evidence that PDL cells possess the potential to be regulatorily involved in periodontal hard tissue repair by at least two distinct mechanisms. One mechanism includes PTH-induced modifications of the osteoprotegerin/receptor activator of nuclear factor κB ligand (OPG/RANKL) ratio resulting in altered osteoclast differentiation and activation, and the second comprises the PTH effect on PDL cell proliferation, differentiation and survival.

The rationale for the present investigation was to clarify further the latter assumption by examining whether changes in PDL cell number in response to PTH(1-34) result from altered proliferation and/or modified apoptotic signaling. We hypothesized that the maturation state of the cells and the mode of PTH(1-34) administration would have an impact on the cellular response.

We also speculated that PTH(1-34) would affect nitric oxide (NO) production by PDL cells. NO produced by NO synthase activates intracellular soluble guanylate cyclase to produce intracellular cyclic guanosine 3′–5′-monophosphate, which triggers rapid cellular responses such as cell proliferation, differentiation and apoptosis under physiological conditions (11–13). Both NO and NO synthase were reported to be expressed in periodontal tissues (14,15). NO expression in these tissues is altered by mechanical stress (16) and the inhibition of NO synthase results in a reduction of experimental tooth movement *in vivo* (17,18). As NO was implicated in mediating both pro- and anti-apoptotic effects in several *in vitro* culture model systems (12,19), we also wanted to elucidate the role of NO as a potential mediator of PTH(1-34)-induced apoptotic signaling in PDL cells.

Material and methods

Cell culture and PTH administration

Human PDL cells were scraped from the middle third of the roots of premolars from six different human donors, aged between 12 and 14 yrs, which showed 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. Fourth-passage cells were plated onto 96- and 24-well plates ($n = 6$), respectively, such that, at harvest, they reached a preconfluent ($\approx 70\%$), a confluent or a postconfluent state. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) 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.

Preliminary experiments in our laboratory did not show any significant dose-dependence of the PTH effect in PDL cells. Thus, to assess whether PTH acts differently at different stages of maturation, as noted by Isogai *et al.* (20), preconfluent, confluent and postconfluent cells were cultured in the presence of 10^{-12} M PTH(1-34) (Sigma-Aldrich, Seelze, Germany) for 0, 1 and 24 h within a 48-h incubation cycle. For the remaining time, experimental media were replaced with tissue culture media without PTH(1-34). These cycles were carried out three times. 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 ensure that bioactive PTH(1-34) was present continuously in the culture system. Ethanol-treated cultures for each treatment group served as vehicle controls.

Cell number

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 DMEM containing 10% FBS. Thereafter, the cell suspension was centrifuged and the cell pellet resuspended in 0.9% NaCl. Finally, the 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.

Proliferation assay

Cell proliferation was determined by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation into the DNA of proliferating cells. The incorporation of the BrdU into DNA over a time period of 4 h was detected by immunoassay (Roche, Mannheim, Germany). The absorbance was read with an enzyme-linked immunosorbent assay (ELISA) reader at 450 nm and 690 nm as a reference wavelength.

DNA fragmentation assay

DNA fragmentation in cell lysates was determined using a commercially available kit (Roche). The assay is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of cell lysates. The amount

of nucleosomes retained in the immunocomplex was determined photometrically with 2,2′-azino-bis-3-ethylbenzthiazoline-sulfonate (ABTS) as substrate at 405 nm and 490 nm as a reference wavelength.

NO production

NO accumulation in the conditioned media was determined using the Griess reagent system (Promega, Mannheim, Germany). Briefly, 100 µl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% phosphoric acid) (Sigma-Aldrich) was mixed with 100 µl of conditioned media in the wells of a 96-well plate. The mixture was incubated for 10 min at room temperature, and the absorbance at 570 nm was measured on an enzyme-linked immunoassay plate reader. The concentration of NO was then determined from a standard curve, with use of sodium nitrite (Sigma-Aldrich) as a standard.

Determination of the duration of the PTH effect on DNA fragmentation

In order to determine the duration of the PTH effect on DNA fragmentation in preconfluent, confluent and postconfluent cells, PDL cells were challenged with 10^{-12} M PTH(1-34) starting 1 h before exposure of the cells to the pro-apoptotic agent, etoposide (5×10^{-5} M) (Sigma, Seelze, Germany). DNA fragmentation was assayed 6 and 24 h after the addition of this agent.

Statistical analysis

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

Results

Vehicle-treated cultures for each experimental group at a particular maturation state did not differ significantly from each other and therefore in all of the figures only one-vehicle treated control for each maturation state is presented.

At the beginning of exposure to PTH(1-34), the cell number in preconfluent PDL cells was $0.06 \pm 0.006 \times 10^5$ cells/well, $0.39 \pm 0.019 \times 10^5$ cells/well in confluent cells, and $0.66 \pm 0.048 \times 10^5$ cells/well in postconfluent cells.

Intermittent PTH(1-34) challenge reduced the cell number significantly in preconfluent PDL cells and this effect was further pronounced when PTH(1-34) was present throughout the entire culture period. In contrast, there was a significant increase in the cell number in confluent and postconfluent PDL cells in response to intermittent PTH(1-34) for 24 h/cycle, although the cell number increased at a reduced rate in postconfluent cells compared with confluent cells. Continuous PTH(1-34) exposure of confluent and postconfluent cultures resulted in a drop in the cell numbers below those observed for the vehicle-treated controls (Fig. 1).

When the cultures were assayed for BrdU incorporation as a marker of proliferation, there was an increase in proliferative activity in preconfluent cells with a maximum following intermittent PTH(1-34) exposure for 24 h, whereas continuous PTH(1-34) challenge did not alter BrdU incorporation of the cells. In vehicle-treated confluent and postconfluent cultures, proliferation was generally reduced compared with preconfluent cells, and intermittent PTH(1-34) for 1 h/cycle further reduced the proliferative signal. Following intermittent PTH(1-34) for 24 h/cycle, BrdU incorporation increased above control levels. In confluent cells, a continuous treatment regimen resulted in a proliferation rate similar to that of the vehicle-treated control, whereas in postconfluent cells, continuous PTH(1-34) exposure led to a decrease in proliferation, as observed

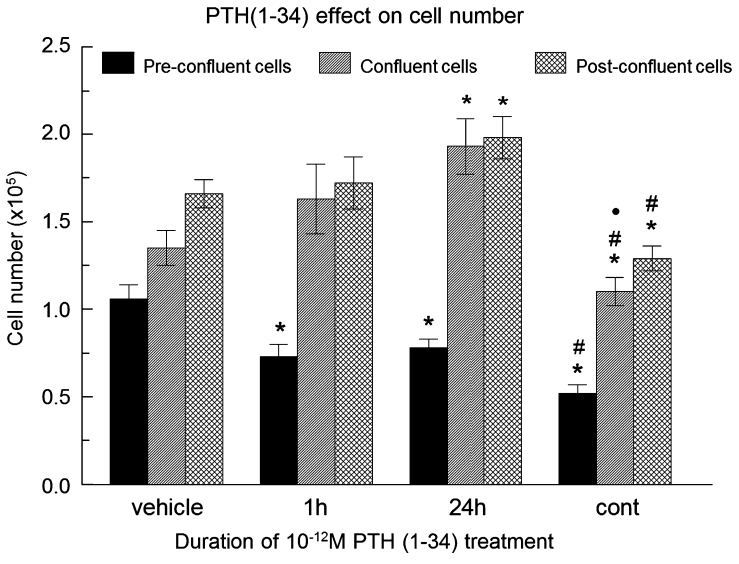

Fig. 1. Effect of parathyroid hormone [PTH(1-34)] treatment on the regulation of cell number in preconfluent, confluent and postconfluent periodontal ligament (PDL) cells. Cells were either treated intermittently with 10^{-12} M PTH(1-34) for 0, 1 or 24 h during three cycles of 48 h each, or challenged continuously (Cont). At harvest, they were counted using a cell counter. Data were acquired from one of two separate experiments, both yielding comparable results. Each value represents the mean ± standard error of the mean (SEM) of six independent cultures. $*p < 0.05$, experimental group vs. vehicle-treated control at a particular maturation state; $\#p < 0.05$, experimental group vs. PTH(1-34) for 24 h/cycle at a particular maturation state.

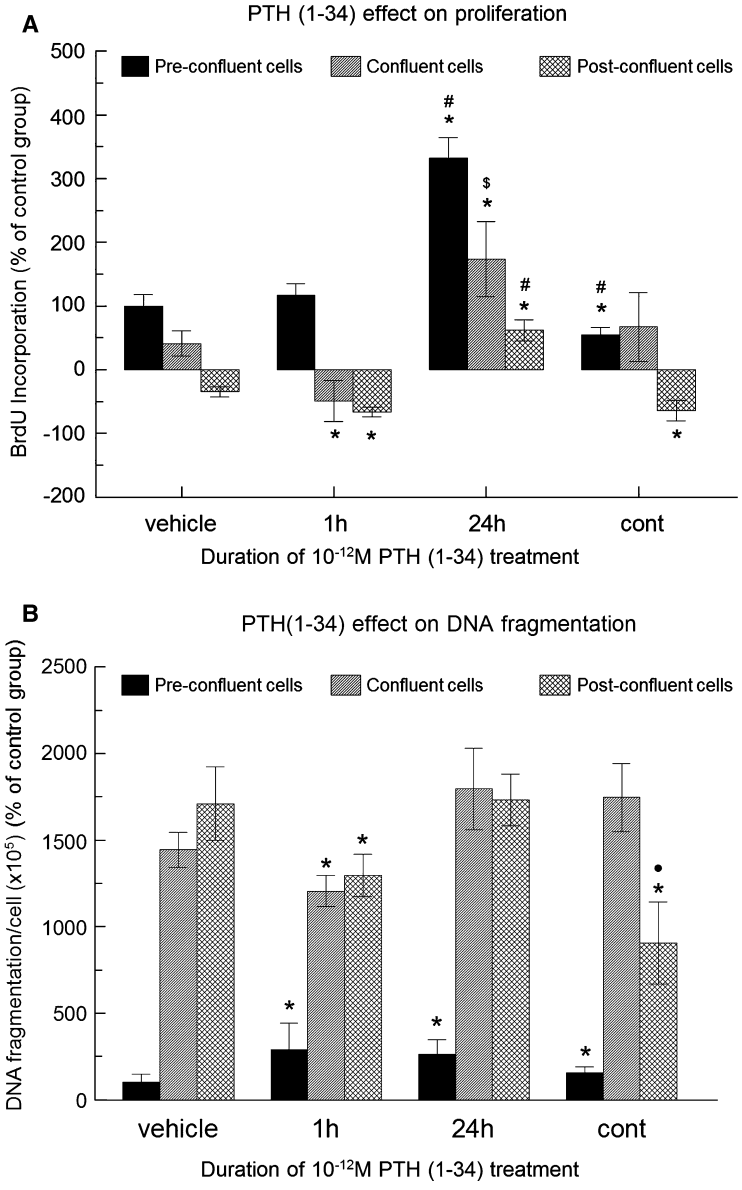

Fig. 2. Parathyroid hormone [PTH(1-34)] effect on proliferation (A) and DNA fragmentation (B) as determined by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation and determination of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of cell lysates, respectively. Cells were either treated intermittently with 10^{-12} M PTH(1-34) for 0, 1 or 24 h during three cycles of 48 h each, or were challenged continuously (Cont.). From all data obtained, the adsorption at the onset of PTH(1-34) administration (T_0) was subtracted and the mean value for the vehicle-treated preconfluent control group was set to 100% and served as a reference. Data were acquired from one of two separate experiments, both yielding comparable results. Each value represents the mean ± standard error of the mean (SEM) for six independent cultures. *$p < 0.05$, experimental group vs. vehicle-treated control; #$p < 0.05$, experimental group vs. other experimental groups at the same state of maturation; •$p < 0.05$, experimental group vs. PTH(1-34) for 24 h/cycle; \$$p < 0.05$, experimental group vs. PTH(1-34) for 1 h/cycle.

after intermittent PTH(1-34) for 1 h/cycle (Fig. 2A).

Besides proliferation, PTH(1-34) also significantly affected DNA fragmentation in PDL cells. In preconfluent cells, DNA fragmentation increased by 188% following 1 h/cycle PTH(1-34) and by 163% after 24 h/cycle PTH(1-34) compared with the vehicle-treated control. Continuous PTH(1-34) enhanced DNA fragmentation by 55%. The basal levels of DNA fragmentation were generally higher in confluent and postconfluent cells, and the DNA fragmentation by PTH(1-34) still remained far higher than the levels detected in preconfluent cells. Intermittent PTH(1-34) for 1 h/cycle reduced DNA fragmentation in confluent and postconfluent cells, whereas PTH(1-34) for 24 h/cycle further enhanced apoptosis at both maturation states. Continuous PTH(1-34) increased apoptosis in confluent cells but decreased DNA fragmentation in postconfluent cells (Fig. 2B).

Intermittent exposure of preconfluent cells to PTH(1-34) resulted in a gradual decrease of NO production, although these changes displayed a trend and were not statistically significant. Continuous PTH(1-34) also reduced NO production, to a level similar to that observed with 24 h/cycle PTH(1-34). In contrast, PTH(1-34) did not alter NO protein expression in confluent cells, regardless of the mode of administration (Fig. 3).

To examine the duration of the PTH(1-34) effect on DNA fragmentation, apoptosis was induced by the pro-apoptotic agent etoposide in the presence of PTH(1-34). In preconfluent cells, etoposide did not alter the apoptotic activity significantly when administered for 6 h but, after 24 h, a significant increase in DNA fragmentation was detectable and this effect could not be antagonized or reduced by PTH(1-34) (Fig. 4A). In confluent cells, etoposide enhanced DNA fragmentation after 6 h to 165% of the vehicle-treated control but when the cells were cultured in the presence of etoposide and PTH(1-34), DNA fragmentation decreased to 71% of the vehicle-treated control. After 24 h, the apoptosis-inductive effect of etoposide was more pronounced and could not be inhibited by PTH(1-34) (Fig. 4B). Similar results were obtained in postconfluent cells. It is remarkable that in the cultures treated with etoposide and PTH(1-34) for 24 h, PTH(1-34) enhanced the pro-apoptotic effect of etoposide alone (Fig. 4C).

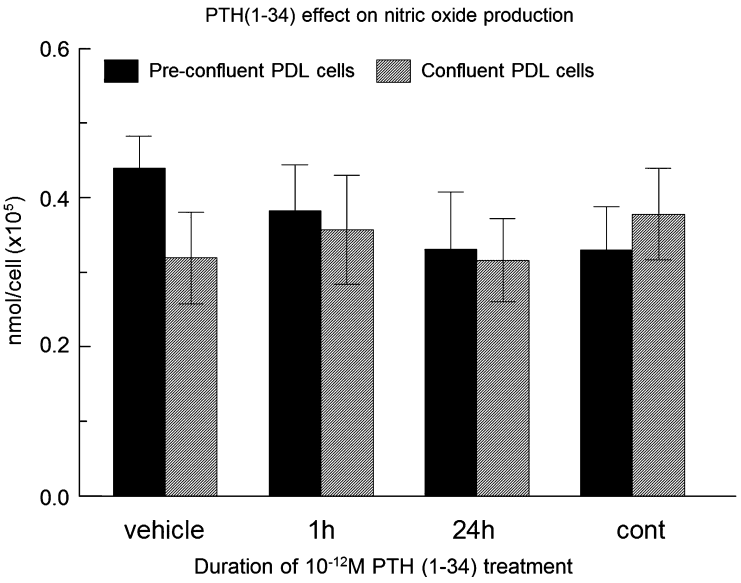

Fig. 3. Influence of parathyroid hormone [PTH(1-34)] administration on nitric oxide (NO) produced by human periodontal ligament (PDL) cells. Cells were either treated intermittently with 10^{-12} M PTH(1-34) for 0, 1 or 24 h during three cycles of 48 h each, or challenged continuously (Cont.). The gradual decrease in NO production observed in preconfluent cells displayed a trend but did not prove to be statistically significant. Data were acquired from one of two separate experiments, both yielding comparable results. Each value represents the mean ± standard error of the mean (SEM) of six independent cultures.

Discussion

Previous studies have demonstrated that exposure to low-dose PTH results in increased proliferation of osteoprogenitor cells (21). This is in line with our observations of increased cell number as a result of enhanced proliferation, together with reduced, or at least unaffected, apoptosis in PTH-challenged confluent and postconfluent PDL cell cultures. Although the proliferative signaling was enhanced in response to PTH(1-34) in preconfluent cells, the cell numbers decreased in these cultures. At the same time, DNA fragmentation was also enhanced in preconfluent cells. Thus, PTH(1-34) might have shifted the balance between proliferation and apoptosis, resulting in a dominance of apoptosis over mitosis, leading to decreased cell numbers. Similar observations were made when actively proliferating cells in young rats were labeled with BrdU and those rats were treated with PTH for 3–5 d. The percentage of BrdU-labeled cells increased but in this same time interval, the percentage of apoptotic cells also increased transiently (22). The simultaneous increase in proliferation and apoptosis is not surprising because proliferation was found to be inevitably associated with apoptosis through the actions of tumor suppressor genes, such as p53, which control key stages of the cell cycle to ensure that cells in which DNA becomes significantly flawed are eliminated through cell death (23,24).

Data presented on osteoblasts indicated that the PTH effect on the cellular response in general, and on apoptosis in particular, depends strongly on the cell status (6,20). In the study by Chen and co-workers, PTH promoted cell viability in preconfluent cells while reducing viability in confluent cells. These findings conflict with our results in which PTH(1-34) enhanced DNA fragmentation in preconfluent cells but did not affect apoptosis in more mature cultures. Different culture conditions, treatment modalities, cell density and the source of the cells employed, might account for these apparent discrepancies.

Besides the maturation-state of the cells, the mode of PTH(1-34) administration was also shown to be important for apoptotic signaling in osteoblasts. Intermittent PTH was found to reduce the prevalence of osteoblast apoptosis, whereas sustained elevation of PTH does not; instead, it rather enhances osteoclastogenesis (25). We did not observe any adverse effects of continuous, compared with intermittent, treatment with PTH(1-34). Ma and co-workers (26) observed the effects of continuous PTH as early as 1 h in a rat model, with a peak after 6 h. The measurements in the present study were obtained after a culture period of up to 10 d. Although PTH receptor levels and responsiveness of the receptor to stimulation appear to increase during osteoblastic phenotypic maturation *in vitro* (27–29), sustained PTH stimulation for a culture period of 10 d might lead to a down-regulation of the number or sensitivity of PTH receptors in PDL cells as part of a feedback mechanism. This might explain our results, at least in part. Another technical consideration refers to the way that PTH(1-34) is administrated continuously. In traditional *in vitro* model systems, unless the wells are continuously perfused, the cells see the PTH once and by the time the receptors recycle, the remaining PTH is probably no longer active as a result of degradation or oxidation. In the experiments of the present study, we refreshed the experimental media containing PTH(1-34) in the continuously exposed groups every 2 d to overcome the limitation of the traditional model systems.

We show that the anti-apoptotic effect of PTH(1-34) in PDL cells is short-lived because PTH(1-34) inhibited etoposide-induced apoptosis after 6 h, but this effect was no longer evident after 24 h, although the hormone was present during the entire period of the experiment. This phenomenon might be explained by a self-limiting effect of the hormone resulting from receptor desensitization. Other studies support this probable mechanism and, in addition, have presented data indicating that the short-term duration of PTH-induced attenuation of apoptosis is not a result of loss of PTH bioactivity but caused by the proteasomal degradation of Runx2, which is neces-

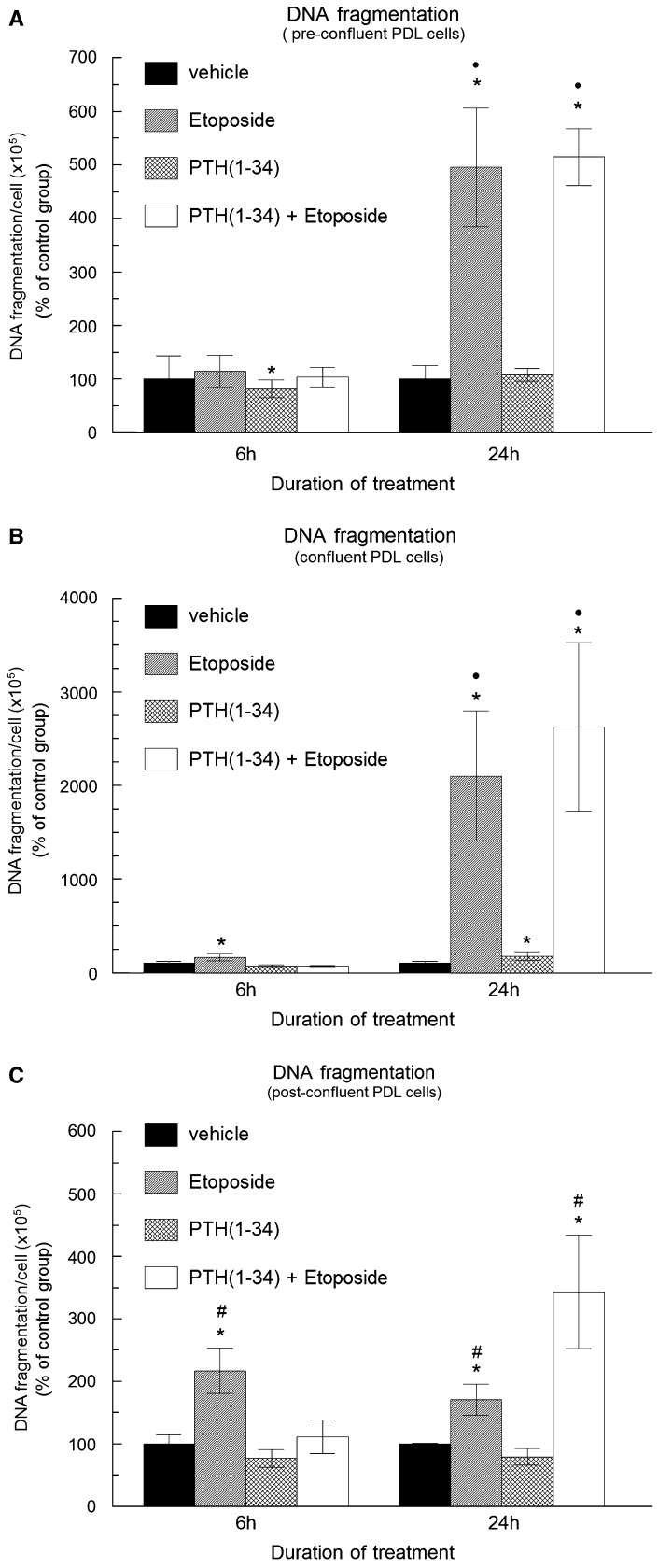

Fig. 4. Duration of the parathyroid hormone [PTH(1-34)] effect on DNA fragmentation in preconfluent (A), confluent (B) and postconfluent (C) periodontal ligament (PDL) cells. The cells were exposed to 10^{-12} M PTH(1-34) for 1 h prior to the addition of the pro-apoptotic agent, etoposide, at a concentration of 5×10^{-5} M. After another 6 or 24 h, respectively, cells were harvested and assayed for DNA fragmentation by using an enzyme-linked immunosorbent assay (ELISA). Data were acquired from one of two separate experiments, both yielding comparable results. Each value represents the mean ± standard error of the mean (SEM) of six independent cultures. $*p < 0.05$, experimental group vs. vehicle-treated control; $\#p < 0.05$, experimental group vs. the other experimental groups at the same time point; $\bullet p < 0.05$, experimental group vs. the PTH(1-34)-treated group.

sary for the transcription of certain survival genes, such as Bcl-2 (25).

Although the effects of NO on osteoblastic function are still not completely understood, there is evidence that a slow and moderate release of NO stimulates the replication of primary rat osteoblasts and alkaline phosphatase activity, whereas a rapid release and high concentrations of NO inhibit proliferation and induce apoptosis (19,30,31). We sought to determine the role of NO as a potential mediator of the PTH(1-34) effect on proliferation and also on DNA fragmentation in PDL cells. In our study, NO was constitutively expressed at low basal levels by PDL cells at different maturation states, suggesting a regulatory role for this free radical under physiological conditions. Constitutive NO can be an effective mediator to regulate proliferation and differentiation in osteoblasts (32) and might contribute to the maintenance of PDL integrity or act as a paracrine and autocrine mediator of PDL cells in response to diverse stimuli, such as pro-inflammatory cytokines (33), mechanical strain (34) and sex hormones (35). However, these hypotheses were not addressed in the present study. NO production was not affected by PTH(1-34) and this matched findings in mouse primary osteoblastic cells where it

required the simultaneous action of mechanical stress for PTH to influence NO levels (36).

In conclusion, PTH(1-34) modifies the balance between proliferating and apoptotic human PDL cells in a maturation state-dependent manner. Besides the PTH(1-34) effect on PDL cell differentiation and local factor production, the present findings provide a further possible mechanism to support the idea that PDL cells possess the potential ability to be involved in the regulation of dental hard tissue repair. The results of this study contribute to a better understanding of the cell-regulatory mechanisms underlying regenerative processes following inflammatory periodontal disease or tooth root resorption processes. In addition, PDL cells might represent a potentially interesting target in order to influence reparative processes pharmacologically. Intermittent PTH(1-34) application might prove beneficial to support the osteointegration of dental implants, prevention of ankylosis following tooth transplantation and formation of reparative cementum occurring after tooth root resorption induced by orthodontic tooth movement.

Acknowledgements

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