Annals of Anatomy 195 (2013) 455–460

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.de/aanat

Research article

Continuous PTH modulates alkaline phosphatase activity in human PDL cells via protein kinase C dependent pathways in vitro

M. Wolf*, A. Jäger[1], N. Abuduwali[1], W. Götz[1], S. Lossdörfer[1]

Dept. of Orthodontics, Dental Clinic, University of Bonn, Welschnonnenstr. 17, 53111 Bonn, Germany

ARTICLE INFO

Article history:
Received 21 March 2013
Received in revised form 6 April 2013

Keywords:
Parathyroid hormone
Human PDL cells
Alkaline phosphatase activity
PKC
PKA
In vitro

SUMMARY

Periodontal ligament (PDL) cells, a major component of the tooth supporting apparatus, share osteoblastic characteristics including their responsiveness to parathyroid hormone (PTH). Clinical studies have already pointed to the benefit of PTH in supporting regenerative processes in the craniofacial region. However, those reports did not analyze which cells mediated the PTH effect on the alveolar bone. The aim of the present study has been to further elucidate the mechanism of action of continuous PTH application on human PDL-cells mimicking a local bolus application and to analyze its intracellular signalling pathways to widen the theoretical basis for future development of reliable local PTH delivery protocols.

Analyses of PDL of extracted human teeth as well as cultured human PDL-cells demonstrated strong expression of PTH-receptor-1 by immune fluorescencecytochemistry/histochemistry. To examine the effect of short time continuous PTH treatment on PDL-cell osteogenic differentiation, PDL-cells were stimulated for 48 h. Analyses for mRNA and protein expression of the early osteogenic marker alkaline-phosphatase revealed an enhanced expression. Pathways analyses mediating the PTH effect resulted in a similar effect when PDL-cells were stimulated with either the signal specific fragments lacking the PKA-activating domain PTH(3–34), PTH(7–34), second-messenger-analogues PKC (PMA) or inhibitors for PKA (H8). Inhibition of the PKC-dependent pathway by stimulation with PTH(1–31), PKA second-messenger-analogue (forskolin) or PKA-inhibitor (RO-32-0432) abolished the PTH effect

These data indicate abundant expression of PTH1R within the PDL and a stimulatory effect of short time continuous PTH on PDL cell differentiation towards an osteogenic phenotype and suggested local PTH application protocols as a possible treatment option in periodontal therapy.

1. Introduction

Periodontal destruction is one of the most prevalent diseases in man (Flores et al., 2008). The disease pattern is multi-faceted, ranging from non-infectious tooth root resorption as a side effect of orthodontic treatment to severe inflammatory periodontitis. Both circumstances are characterized by the degradation of dental connective tissues such as alveolar bone, cementum and the periodontal ligament and further progression will eventually result in compromised support or even loss of teeth (Nakahara et al., 2004; Pihlstrom et al., 2005). Thus, the desirable goal of current periodontal treatment approaches is the regeneration of the physiological periodontal architecture, including the attachment of new connective tissue to the previously denuded or resorbed root surface along with the formation of functional connective collagen fibres, cementum and alveolar bone (Aukhil et al., 1990). Contemporary reconstructive periodontal treatment strategies focus on tissue engineering techniques taking advantage of increasing knowledge from cell biology, biomaterials and medicine to enhance the healing process (Flores et al., 2008). These concepts comprise the use of pro-regenerative growth factors and hormones, but their use is still at an experimental stage (Nakahara et al., 2004; Akizuki et al., 2005; Hasegawa et al., 2005).

These various approaches share the well-accepted concept of remnant PDL cells at the injured site being essential for periodontal regeneration to occur, since the heterogeneous population of PDL cells hosts all cell types required for the synthesis of the different components of the PDL. Among those, cells with stem cell-like properties as well as precursors of the different cell types that are capable of synthesizing cementum, bone and connective tissue have been identified within the PDL. Osteoblast-like characteristics have been attributed to a certain proportion of PDL cells including the expression of osteoblastic marker genes such

* Corresponding author. Tel.: +49 228 287 22433; fax: +49 228 287 22588.
E-mail addresses: michael.wolf@uni-bonn.de, michael.wolf@ukb.uni-bonn.de (M. Wolf), andreas.jaeger@ukb.uni-bonn.de (A. Jäger), serk_omar@yahoo.com (N. Abuduwali), wgoetz@uni-bonn.de (W. Götz), stefan.lossdoerfer@ukb.uni-bonn.de (S. Lossdörfer).
[1] Tel.: +49 228 287 22433; fax: +49 228 287 22588.

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http://dx.doi.org/10.1016/j.aanat.2013.04.006

as alkaline phosphatase (ALP) and osteocalcin, collagen synthesis, and a comparable response to osteoinductive stimuli (Basdra and Komposch, 1997; Nohutcu et al., 1997; Isaka et al., 2001; Ivanovski et al., 2001; Seo et al., 2004). From in vitro and in vivo experiments, it can be concluded that PDL cells are able to form mineralized nodules under culture conditions appropriate for osteo differentiation (Ouyang et al., 2000; Lekic et al., 2001; Wolf et al., 2012b). Combining these lines of evidence which point to a regulatory role for PDL cells in the orchestration of periodontal repair processes led us to focus our research interest on anabolic treatment regimens to support the regenerative capacity of PDL cells.

Within the increasing list of pharmaceutical agents to affect bone metabolism, parathyroid hormone (PTH) is currently the only FDA approved anabolic agent in the U.S. for regeneration of bony tissue (Pettway et al., 2008). Depending on the mode of its administration, exogenous PTH may exert anabolic or catabolic effects on bony tissues (Ishizuya et al., 1997; Datta and Abou-Samra, 2009). The ability of anabolic doses of PTH to increase parameters of bone formation raised the interest for its applicability in the orofacial region to support periodontal repair processes. Preclinical studies have suggested that osseous lesions in the oral cavity may be a receptive target for the anabolic actions of PTH (Barros et al., 2003; Jung et al., 2007; Bashutski et al., 2010; Wolf et al., 2012b). A recent clinical investigation by Bashutski et al. demonstrated that intermittent PTH treatment improved clinical outcomes of patients after periodontal surgery as evidenced by a significant reduction of alveolar bone defects, accelerated wound healing and a gain of clinical attachment level (Bashutski et al., 2010). Despite broad acknowledgement of those findings, this study could not elucidate which cells actually mediated the PTH effect. Therefore, prior to an extension of the range of clinical application, a deeper understanding of the mechanism of action of PTH particularly on PDL cells has to be developed. This includes the need for an elucidation of the intracellular signalling pathways involved in the mediation of both the effect of intermittent and continuously applied PTH. Although the continuous action of the hormone is generally thought to be catabolic and of no benefit for regenerative treatment approaches, in-depth knowledge on its mechanism of action in PDL cells is crucial.

In studies on osteoblasts it has been established that, upon binding to its G-protein coupled receptor (PTH1R), PTH function is regulated via protein kinase A (PKA) and protein kinase C (PKC) pathway and leads via phosphorylation of specific transcription factors to transcriptional activity of genes in the cell nucleus, including ALP as a marker for early osteoblastic differentiation and osteocalcin indicating late osteoblastic differentiation events (Datta and Abou-Samra, 2009). In a possible clinical scenario, one conceivable method of PTH administration would be a local bolus injection in periodontal tissues or its placement into scaffolds within the tooth supporting apparatus. In this case, PTH would be present continuously in the local microenvironment until degradation. However, despite all information available on continuous systemic PTH treatment, the effect of such a short-term cPTH administration on PDL cell differentiation still remains to be elucidated.

Therefore, the aim of the present investigation was to demonstrate the expression of the PTH1R in human PDL cells and tissue. Furthermore, we hypothesized that a short-term application of cPTH, mimicking a single shot bolus application in the periodontal space for therapeutic use, induces PDL cell differentiation towards an osteogenic phenotype and would provide a possible treatment option in anabolic regenerative treatment strategies. We speculated that PTH-induced changes in alkaline phosphatase specific activity would be mediated via PKA- or PKC-dependent pathways in PDL cells.

2. Material and methods

2.1. PDL cell culture

To avoid contamination with gingival or apical tissue, human PDL cells were explanted from the middle third of the tooth root from extracted premolars of three different adolescent donors (12–14 years of age) as described previously (Lossdorfer et al., 2005). Extractions of teeth were performed for orthodontic reasons, with written parental consent and following an approved protocol of the ethics committee of the University of Bonn.

Fifth passage PDL cells of the three donors were seeded into 24-well plates ($n = 6$) at a density of 20,000 cells/well and cultured to subconfluence (~70%). Cells were cultured in DMEM containing 10% foetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5000 U/ml penicillin and 5000 U/ml streptomycin; Biochrom AG, Germany) at 37 °C in an atmosphere of 100% humidity, 95% air, and 5% CO_2. Prior to experimental use, cells were characterized for their mesenchymal origin as described previously (Lossdorfer et al., 2011).

2.2. Human periodontal tissue

Five extracted human teeth were fixed in with 4% paraformaldehyde for fixation purposes and prepared for light microscopical examination as recently described (Gotz et al., 2006; Wolf et al., 2012a). All specimens were decalcified in neutral 10% ethylene diamine tetra-acetic acid before processing for paraffin histology. To perform immune histochemical analyses, 5–7 μm serial sagittal sections were prepared and stained according to standard protocols using a 1:50 dilution of the monoclonal primary antibody (Santa Cruz Biotechnology, CA, USA; concentration: 200 μg/ml; incubation at 4 °C overnight) followed by a HRP-conjugated goat anti-mouse secondary antibody (Dako EnVision™+ System-HRP (DAB); ready-to-use solution, Dako, Hamburg, Germany) for 1 h at room temperature. After washing for 10 min with TBS, freshly prepared DAB substrate was added to the slides until a suitable staining developed. The sections were rinsed with water and counterstained with Mayer's haematoxylin. Negative controls were included in each experiment to verify antibody specificity, using a non-specific IgG antibody (Santa Cruz Biotechnology, CA, USA). Sections of selected tissues carrying significant amounts of the PTH1R antigen (human bone) were treated in the same way as the tooth sections and served as positive controls (data not shown). The same protocol was applied to PDL cell cultures for immunocytochemical demonstration of PTH1R expression.

2.3. In vitro experiments, PTH administration and real time PCR

To analyze the time course of the PTH effect on ALP mRNA expression with an expected peak occurring earlier at the transcriptional level than at the protein level, fifth passage PDL cells were exposed to 10^{-9} M PTH(1–34) for 1 h, 3 h, 6 h. Vehicle treated cultures served as controls. At harvest, gene expression level of alkaline phosphatase was analyzed by real time PCR using the iQ™ SYBR Green Supermix (Biorad, Germany) according to the manufacturer's instructions on a light cycler (Roche, Germany) as described previously (Lossdorfer et al., 2010; Kraus et al., 2012). All primers were verified by computer analysis for specification using the basic logical tool (BLAST) and synthesized at high quality (Metabion, Germany).The primer sequences and conditions (annealing temperature 69°) used were as published previously (Kraus et al., 2012): ALP sense 5′ AGA-GAA-AGC-GAT-GGT-GGA-TG 3′, antisense 5′ CGG-TGG-CAT-TAA-TAG-TGA-GAT-G 3′. β-actin was used as an endogenous reference sense: 5′-CATGGATGATGATATCGCCGCG-3′, antisense: 5′-ACATGATCTGGGTCATCTTCTCG-3′.