J Periodont Res 1996; 31: 369–372
Printed in Denmark · All rights reserved

JOURNAL OF
PERIODONTAL RESEARCH
ISSN 0022-3484

Short communication

Expression of mineral-associated proteins by periodontal ligament cells: *in vitro* vs. *ex vivo*

R. M. Nohutcu[1], L. K. McCauley[2], Y. Shigeyama[3], M. J. Somerman[2,4]

[1]Hacettepe University, Faculty of Dentistry, Department of Periodontology, 16100 Ankara, Turkey; [2]Department of Periodontics/Prevention/Geriatrics, University of Michigan, Ann Arbor; MI; [3]Department of Periodontology, Osaka Dental University, Osaka,Japan; [4]Department of Pharmacology, University of Michigan, Ann Arbor, MI, USA

Nohutcu RM, McCauley LK, Shigeyama Y, Somerman MJ: Expression of mineral-associated proteins by periodontal ligament cells: in vitro *vs* ex vivo. *J Periodont Res 1996; 31: 369–372.* © Munksgaard, 1996

Martha Somerman, Department of Periodontics/Prevention/Geriatrics, University of Michigan, 1011 N. University Ave, Ann Arbor, Michigan 48109-1078, USA

Key words: gene expression; bone; periodontal ligament; cell culture

Accepted for publication December 5, 1995

While several research groups have focused on establishing the role of periodontal ligament (PDL) cells in the local environment during periodontal wound healing, *in vivo* and *in vitro* (see reviews 1, 2), questions remain regarding the "osteoblast-like" properties of PDL cells. Recent results from our laboratory prompted us to query whether cultured PDL cells express mRNA for mineral-associated proteins in the same manner as freshly extracted tissues. First, we noted that bone sialoprotein (BSP) was expressed in cells lining the root surface during molar root development and in developed roots in mice *in situ* (3), but expression in cultured PDL cells was minimal. Secondly, exposure of PDL cells to dexamethasone, an agent which promotes osteoblast differentiation, resulted in a significantly greater parathyroid hormone (PTH)-mediated cAMP response, when compared with untreated cells (4). These studies suggest that *in vitro* cultures of PDL are selective for a more "fibroblast-like" cell, as seen with many other cell types with passage, *in vitro*, e.g. chondrocytes and osteoblasts (5). However, definitive data to demonstrate differences in protein expression in cultured vs. freshly extracted PDL cells have not been provided.

The purpose of the present investigation was to determine whether freshly extracted PDL cells express mRNA for proteins associated with mineralized tissues, and to compare steady state mRNA levels from freshly extracted cells with cultured PDL cells.

Materials and methods

Tissue isolation and culture

Porcine anterior teeth and mandibular bone were extracted immediately after sacrifice of animals, at a local abattoir. These extractions were performed on 3 separate occasions. PDL fibroblasts were isolated and cultured as reported in detail previously (4). The mandibular bone included portions of cortical and trabecular bone rinsed free of marrow. Comparable PDL tissues were either used for RNA extraction or for explantation. For explantation the PDL tissues were cut into small pieces, rinsed with biopsy media (DMEM with 10% FBS, 250 μg/ml Amphotericin B, 5 μg/ml Gentamicin Sulfate, 100 units/ml penicillin, 100 μg/ml streptomycin; Gibco BRL, Gaithersburg, MD), placed in tissue culture dishes and glass cover slips were placed over the tissue to prevent floating. After a 24 h attachment period the biopsy medium was replaced with culture medium (DMEM with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin). When cells around the explants reached confluence, they were

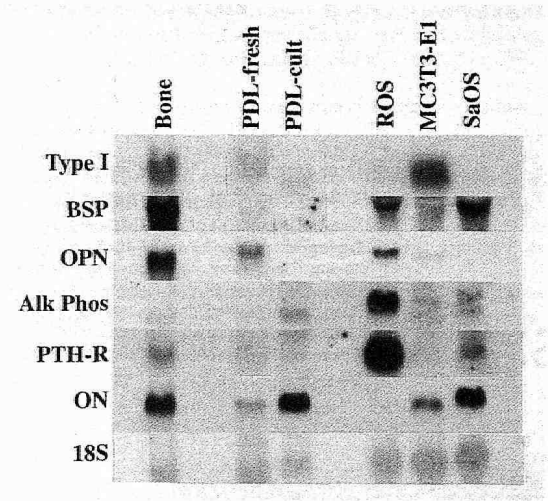

Fig. 1. Autoradiograph of Northern blot analysis for type I collagen (Type 1), bone sialoprotein (BSP), osteopontin (OPN), alkaline phosphatase (Alk Phos), PTH/PTHrP receptor (PTH-R), osteonectin (ON), and 18S rRNA (18S) in total RNA samples from alveolar bone (Bone), periodontal ligament tissue freshly extracted from porcine teeth (PDL-fresh) and periodontal ligament cells extracted from porcine teeth and cultured *in vitro* (PDL-cult), rat osteosarcoma cells (ROS), mouse calvarial pre-osteoblastic cells (MC3T3-E1), and human osteosarcoma cells (SaOS). This is a representative blot where similar results were obtained from tissues obtained on 3 separate occasions.

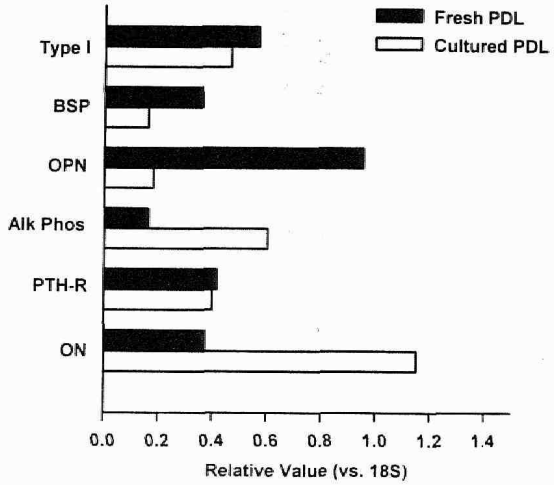

Fig. 2. Relative values for signal intensity of fresh PDL and cultured PDL samples from autoradiograph in Fig. 1. Scanning densitometry was performed and relative values expressed in relation to 18S rRNA signals.

passaged with 0.25% trypsin-0.1% EDTA and used at third passage for RNA extraction.

RNA extraction and Northern blot analyses

Total RNA was extracted from freshly harvested PDL, bone tissue, and cultured PDL cells obtained from animal tissues on 3 separate occasions. In addition, total RNA was isolated from ROS 17/2.8 rat osteosarcoma, SaOS-2 human sarcoma and MC3T3-E1 mouse pre-osteoblastic cells and evaluated adjacent to porcine tissues as standards for osteoblast specific gene expression and to ensure that the probes were adequate for detection of specific RNAs. The fresh PDL and bone were pulverized in ice-cold guanidinium isothiocyanate (4 M) with a mortar and pestle, cell culture cells were extracted from the tissue culture flask with the addition of guanidinium isothiocyanate followed by vortexing. Total RNA was isolated using guanidinium isothiocyanate, phenol and chloroform extraction, quantitated by spectroscopy at 260 nm, electrophoresed (20 µg) on 1.2% agarose formaldehyde gels, and transferred to nylon membranes as previously described (6, 7). The following cDNA probes were hybridized with the immobilized RNA: mouse collagen type I (α_2I) (8), rat parathyroid hormone/parathyroid hormone related protein receptor (PTH/PTHrP receptor; PTH-R) (9), mouse alkaline phosphatase (AP) (10), human osteopontin (OPN) (11), mouse bone sialoprotein (BSP) (12) and rat osteonectin (ON) (13). All cDNA probes were labeled with ^{32}P-dCTP (NEN Dupont, Boston, MA) using random primer labeling (Stratagene Inc., La Jolla, CA), nylon membranes were hybridized, washed and exposed as described (8). Blots were also stripped and reprobed with a cDNA probe for 18S rRNA as a control for even loading (14). Autoradiographs were evaluated for relative signal density with NIH Image Software on a Macintosh computer and data expressed as a ratio of signal density for probe of interest/18S rRNA.

Results and discussion

Representative Northern blots for steady state mRNA levels of ON, OPN, AP, PTH-R, type I collagen and BSP in freshly isolated bone and PDL tissue, and cultured PDL, ROS 17/2.8, SaOS-2, and MC3T3-E1 cells are shown in Fig. 1, where similar results were obtained from 3 separate experiments. The relative density (probe of interest vs. 18S rRNA) of the signal for fresh PDL, and cultured PDL was plotted in Fig. 2, to provide a comparison of signal intensities for this representative blot. There was a 3.3-fold and 2.3-fold increase in steady state AP and ON mRNA, respectively, in cultured vs. fresh PDL tissues. In contrast, there was a 5.3-fold reduction in steady state OPN mRNA for cultured vs. fresh PDL tissues. Steady state mRNA lev-

els for type I collagen and PTH/PTHrP receptor were similar in fresh vs. cultured samples. Freshly extracted PDL were positive for BSP mRNA, while cultured PDL cells had 2.3-fold lower mRNA levels.

The results from these studies provide information critical to our understanding of the PDL in mature tissues and important to our continued progress in the development of regenerative materials. First, it is clear that the PDL contains cells that express RNA for proteins associated with mineralized tissues, e.g. bone sialoprotein, osteopontin, alkaline phosphatase, PTH/PTHrP receptor and type I collagen. Secondly, clear differences were noted in RNA levels for proteins obtained from freshly extracted tissues vs. RNA obtained from cells, *in vitro*. For example, RNA expression for BSP and OPN was detectable in cells obtained from freshly isolated tissue, with much lower levels in cultured PDL cells. Importantly, BSP, a mineralized tissue specific adhesion molecule, is considered to have a role in the initiation of mineralization (15). Osteopontin is an adhesion molecule associated with mineralized tissues, but also identified in several other tissues, including the PDL (16 and references sited). Suggested functions for OPN in mineralized tissues include roles as a regulator of osteoclast activity, and of early stages of mineralization (see review, 17). In addition, higher levels of RNA for osteonectin (ON) were found in cultured PDL cells compared with freshly extracted RNA from comparable cells. This is in agreement with previous studies showing that PDL cells, *in vitro,* express ON (18, 19). Although the exact function(s) of ON is not known, it has been called a culture shock protein since increased protein levels are observed in cells *in vitro* (20). RNA levels for the PTH/PTHrP receptor and type I collagen were similar in cultured vs. fresh tissues. Expression of the PTH/PTHrP receptor is associated with active osteoblast-like cells producing a differentiating matrix, both *in vivo* and *in vitro* (21, 22). While no difference in expression for the PTH/PTHrP receptor was noted in our studies it is conceivable that at different stages of maturation, *in vivo* or *in vitro*, PDL cells may exhibit alterations in RNA levels for this protein.

In conclusion, results from this study highlight the need to be cautious in interpretation of data obtained from PDL cells studied *in vitro*. This does not preclude the fact that valuable information can be obtained from *in vitro* studies, but that direct transfer of information from *in vitro* to *in vivo* requires careful and critical analysis of data. Notably, the data here provide evidence that cells within the PDL region, in mature tissue, do contain cells that reflect "osteoblast/cementoblast-like" properties, based on expression of RNA for mineralized tissue associated proteins.

Acknowledgements

Technical assistance by Amy J. Koh and manuscript preparation by Jessica Madow are appreciated. We are grateful to Bain's Packing and Refrigeration Company for allowing us perform extractions and isolations on site. Investigations supported in part by NIH, DK46919 (LKM), Procter and Gamble, Miami Valley (MJS) and Eczacibasi Procter and Gamble, Oral Care Scientific Research and Award Fund – Turkey (RN).

References

1. Pitaru S, McCulloch CAG, Narayanan SA. Cellular origins and differentiation control mechanisms during periodontal development and wound healing. *J Periodont Res* 1994; **29**: 81–94.
2. O'Neal R, Wang H-L, MacNeil RL, Somerman MJ. Cells and materials involved in guided tissue regeneration. *Current Opin Periodontol* 1994: 144–156.
3. MacNeil RL, Berry JE, D'Errico JA, Strayhorn C, Piotrowski B, Somerman MJ. Role of osteopontin and bone sialoprotein in cementogenesis. *Conn Tissue Res* 1995; **33**: 1–7 [323–329].
4. Nohutcu RM, Somerman MJ, McCauley LK. Dexamethasone enhances the effects of parathyroid hormone on human periodontal ligament cells *in vitro*. *Calcif Tiss Int* 1995; **56**: 571–577.
5. Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB. Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvarial I cells. *J Cell Physiol* 1990; **143**: 213–221.
6. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform-extraction. *Anal Biochem* 1987; **162**: 156–159.
7. McCauley LK, Beecher CA, Melton ME, *et al.* Transforming growth factor β regulates steady state PTH/PTHrP receptor mRNA levels and PTHrP binding in ROS 17/2.8 osteosarcoma cells. *Mol Cell Endocrinol* 1994; **101**: 331–336.
8. Liau G, Yamada Y, de Crombrugghe B. Coordinate regulation of the levels of type III and type I collagen mRNA in most but not all mouse fibroblasts. *J Biol Chem* 1985; **260**: 531–536.
9. Abou-Samra AB, Jüppner H, Force T, et al. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci (USA)* 1992: **89**: 2732–2736.
10. Terao M, Studer M, Gianni M, Garattini E. Isolation and characterization of the mouse liver/bone/kidney-type alkaline phosphatase gene. *Biochem J* 1990; **268**: 641–648.
11. Young MF, Kerr JM, Termine JD, et al. cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). *Genomics* 1990; **7**: 491–502.
12. Young MF, Ibaraki K, Kerr JM, Lyu MS, Kozak CA.

Murine bone sialoprotein (BSP): cDNA cloning, mRNA expression, and genetic mapping. *Mamm Genome* 1994; **5**: 108–111.
13. Findlay DM, Fisher LW, McQuillan CI, Termine JD, Young MF. Isolation of the osteonectin gene: evidence that a variable region of the osteonectin molecule is encoded within an exon. *Biochemistry* 1988; **27**: 1483–1489.
14. Renkawitz R, Gerbi SA. Ribosomal DNA of the fly *Sciara coprophila* has a very small and homogenous repeat unit. *Molec Gen Gener* 1979; **173**: 1–13.
15. Hunter GK, Kyle CL, Goldberg HA. Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. *Biochem J* 1994; **300**: 723–728.
16. MacNeil RL, Berry J, D'Errico J, Strayhorn C, Somerman MJ. Localization and expression of osteopontin in mineralized and nonmineralized tissues of the periodontium. *Ann NY Acad Sci* 1995; **760**: 166–176.
17. Denhardt DT, Guo X. Osteopontin. A protein with diverse functions. *FASEB J* 1993; **7**: 1475–1482.
18. Wasi S, Otsuka K, Yao KL, Tung PS, Aubin JE, Sodek J, Termine JD. An osteonectinlike protein in porcine periodontal ligament and its synthesis by periodontal ligament fibroblasts. *Can J Biochem Cell Biol* 1984; **62**: 470–478.
19. Somerman MJ, Young MF, Foster RA, Moehring J, Emm G, Sauk JJ. Characteristics of human periodontal ligament cells *in vitro*. *Arch Oral Biol* 1990; **35**: 241–247.
20. Lane TF, Sage EH. The biology of SPARC, a protein that modulates cell–matrix interactions. *FASEB J* 1994; **8**: 163–173.
21. McCauley LK, Koh AJ, Beecher CA, Cui Y, Decker J, Franceschi RT. Effects of differentiation and transforming growth factor β on PTH/PTHrP receptor mRNA levels in MC3T3-E1 cells. *J Bone Min Res* 1995; **10**: 1243–1255.
22. Lee K, Deeds JD, Chiba S, Un-No M, Bond AT, Segre GV. Parathyroid hormone induces sequential c-fos expression in bone cells *in vivo*: *in situ* localization of its receptor and c-fos messenger ribonucleic acids. *Endocrinology* 1994; **134**: 441–450.