Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 809–814

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2010.01291.x

Short communication

Human cementoblasts express enamel-associated molecules *in vitro* and *in vivo*

J. Nuñez[1], M. Sanz[1], L. Hoz-Rodríguez[2], M. Zeichner-David[3], H. Arzate[2]

[1]Facultad de Odontología, Universidad Complutense de Madrid, España, [2]Laboratorio de Biología Periodontal y Tejidos Mineralizados, Facultad de Odontología, Universidad Nacional Autónoma de México, México and [3]Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, CA, USA

Nuñez J, Sanz M, Hoz-Rodríguez L, Zeichner-David M, Arzate H. Human cementoblasts express enamel-associated molecules in vitro *and* in vivo. *J Periodont Res 2010; 45: 809–814. © 2010 John Wiley & Sons A/S*

Background and Objective: Cementum is a mineralized tissue that facilitates the attachment of periodontal ligament to the root and surrounding alveolar bone and plays a key role in the regeneration of periodontal tissues. The molecular mechanisms that regulate the proliferation and differentiation of cementoblasts, however, have not been elucidated to date. Enamel molecules are believed to regulate cementoblast differentiation and to initiate the formation of acellular extrinsic fiber cementum. The purpose of this study was therefore to isolate and culture human root-derived cells (HRDC) in order to determine whether they are able to express both cementum and specific enamel proteins and subsequently to confirm these findings *in vivo*.

Material and Methods: Human root-derived cells were isolated and expanded *in vitro*. Cells were characterized using RT-PCR, immunostaining, western blotting and by examination of total mRNA to determine the expression of cementum and enamel markers. Human periodontal tissues were also examined for the expression of enamel-related proteins by immunostaining.

Results: We showed that HRDC express mRNA corresponding to ameloblastin *(AMBN)*, amelogenin *(AMEL)*, enamelin *(ENAM)*, tuftelin *(TUFT)* and cementum-associated molecules such as cementum protein 1 *(CEMP1)* and cementum attachment protein *(CAP)*. Western blotting revealed that HRDC express both AMEL and AMBN gene products, as well as the cementum markers CEMP1 and CAP. *In vivo*, we have showed that AMBN and AMEL are expressed by cementoblasts lining cementum, paravascular cells and periodontal ligament cells.

Conclusion: These results suggest that enamel-associated and cementum-associated proteins could act synergistically in regulating cementoblast differentiation and cementum deposition and offer new approaches on how the cementogenesis process is regulated.

Dr Higinio Arzate, Laboratorio de Biología Periodontal y Tejidos Mineralizados, Facultad de Odontología, Universidad Nacional Autónoma de México, Cd. Universitaria, C.P. 04510, México D.F.
Tel/Fax: +52 55 5622 5563
e-mail: harzate@unam.mx

Key words: cementum; cementogenesis; cementum attachment protein; cementum protein 1; amelogenin; ameloblastin

Accepted for publication March 12, 2010

Cementum is a mineralized tissue that facilitates the attachment of periodontal ligament to the root and surrounding alveolar bone and plays a key role in both periodontal development and regeneration of periodontal tissues (1). The molecular mechanisms that regulate the proliferation and differentiation of cells within the periodontal ligament to become cementoblast cells are not yet completely understood. In fact, some authors attribute a common source to cementoblasts and osteoblasts because they share phenotypic and structural features (2). There is also debate whether cementoblasts are a unique cell type or transformed osteoblasts (3). Cementoblasts share

several molecular characteristics with osteoblasts including the production of type I collagen and several noncollagenous proteins such as bone sialoprotein, osteocalcin and osteopontin (4). Cementum, however, does not have the lamellar organization found in bone: it is an avascular tissue; it also lacks innervation and physiological remodelling and does not contain bone marrow. Recently, it has been reported that the periodontal ligament contains multipotent stem cells that have the potential to differentiate into cementoblast-like cells *in vitro* and to generate cementum and periodontal ligament-like tissue *in vivo* (5). The molecular factors that regulate the differentiation of these populations of stem cells are, however, unknown. One of the major difficulties in studying cementoblasts is the lack of specific markers. Recently, two genes encoding cementum proteins have been isolated: cementum attachment protein *(CAP)* and cementum protein 1 *(CEMP1)* (6). They have been demonstrated as cementoblast-specific products and consequently reliable specific markers of these cells.

The clinical importance in understanding the origin, differentiation and behaviour of these cells lies in the fact that regeneration of periodontal tissues lost during periodontitis is based on the formation of new cementum. It is believed that EMD can regulate cementoblast differentiation and initiate the formation of acellular extrinsic fiber cementum (7,8). In fact, a treatment concept based on the application of EMD aimed for new cementum formation and periodontal regeneration has been extensively used in clinical periodontics and its efficacy has been proven in multiple clinical trials. EMD contains enamel proteins, mainly amelogenin (AMEL), and the expression of enamel proteins by cementoblast cells has also been a subject of debate. The expression of ameloblastin (AMBN) during cementum formation has been demonstrated. However, the expression of AMEL and enamelin (ENAM) by cementoblasts has not been completely accepted, again because of the lack of specific markers for these cells. The purpose of this study was to isolate and culture human root-derived cells (HRDC) in order to determine whether they are able to express both cementum and specific enamel proteins and to confirm these findings *in vivo*.

Material and methods

Cell culture

Human root-derived cementoblast cells were isolated from healthy human teeth extracted for orthodontic reasons and according to the protocols approved by the Internal Review Board of the Universidad Complutense de Madrid, España. Teeth were processed immediately after extraction. The culture and expansion of HRDC were performed according the procedures previously published by Nuñez *et al.* (9). Human alveolar bone-derived osteoblasts (HABDC) were also isolated using standard methods previously reported (10). Cultures from both isolates belonging to either second or third passages were used for the experiments described below.

RT-PCR

The expression of *AMEL*, *AMBN*, *ENAM*, tuftelin 1 *(TUFT1)* *CAP* and *CEMP1* at the mRNA level in HRDC and HABDC was established by RT-PCR. Previously published primer sequences and reaction conditions were used to amplify AMEL, AMBN, TUFT, ENAM, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CEMP1 and CAP (11). The reaction products were analyzed by electrophoresis of 15-μL samples in 2% agarose gels containing 0.5 μg/mL of ethidium bromide. The identity of the PCR products was confirmed by sequencing.

Western blot

After it was determined that HRDC express cementum and enamel-associated molecules at the mRNA level, we determined whether the proteins were also expressed. The expression of proteins was analyzed by western blotting. The HRDC and HABDC were cultured as previously described, then collected, centrifuged and dissolved in lysis buffer containing 1% sodium dodecyl sulfate and a protease inhibitor cocktail (8). Equal amounts of total protein (10 μg per lane) were separated by gel electrophoresis, blotted onto Immobilon-P [poly(vinylidene difluoride)] membranes, as described elsewhere (6), and incubated with rabbit antiserum to AMEL, AMBN, or human recombinant cementum protein 1 (diluted 1:500) or with a mouse monoclonal anti-CAP (diluted 1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG secondary antibody or rabbit anti-mouse IgG secondary antibody. Detection was performed as previously described (6).

Immunostaining

Immunoexpression of AMEL, AMBN, CAP and CEMP1 was also determined in HRDC and HABDC cultured *in vitro*. Double-immunofluorescence staining was performed using mouse monoclonal antibody against bovine CAP (Santa Cruz Biotechnology, Inc.), rabbit polyclonal antibody against human CEMP1 and polyclonal antibodies against mouse AMEL and AMBN, as described elsewhere (12). Cells were plated at a low density in eight-well Lab-Tek chamber slides, cultured for 7 d, fixed and incubated with the antibodies against the proteins under study. Negative controls were achieved by omitting the primary antibody or by incubating with normal rabbit or mouse serum. The spatial expression of AMEL and AMBN in human periodontal tissues was analyzed by immunostaining. Human dental tissues were obtained from a autopsy of a 29-year-old man, following the policies of the Research Review Board of the Instituto Nacional de Cancerología, México City. The specimens containing periodontal structures were fixed, decalcified, embedded in paraffin, sectioned and mounted on glass slides (13). Slides were incubated with the corresponding antibodies. Sections incubated with normal rabbit

serum or lacking first antibody were used as controls.

Results

Expression of cementum and enamel-related molecules at the mRNA level

The expression of cementum and enamel-related molecules in HRDC and HABDC was first determined by RT-PCR. Our results show that AMEL, AMBN, ENAM, TUFT1, CAP and CEMP1 amplicons were all expressed by HRDC. However, these amplicons were not detected in reactions using total RNA obtained from HABDC (Fig. 1A).

Expression of cementum and enamel-related molecules at the protein level

Total protein extracted from HRDC and HABDC and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by western blotting was used to determine the expression of cementum and enamel-related proteins The results shown in Fig. 1B indicate that all proteins tested – AMEL (lane 1), AMBN (lane 2), CEMP1 (lane 3) and CAP (lane 4) – revealed a main protein band at 27, 62, 55 and 50 kDa, respectively. Human alveolar bone-derived cells were negative for these proteins (data not shown).

Immunolocalization of the CAP protein in HRDC showed immunoreactivity in the cell cytoplasm and nucleus (Fig. 2A,D,G). Double staining for AMEL, AMBN and CEMP1 demonstrated that CAP co-localizes with enamel-associated proteins and CEMP1 (Fig. 2E,H). Human alveolar bone-derived cells were negative for these proteins (data not shown). Immunodetection of AMEL and AMBN in human sections showed the expression of AMEL in cementoblasts and a slightly positive immunoreactivity in the cementum mineralized matrix (Fig. 3A), mainly in the acellular cementum region (Fig. 3A). Amelogenin expression was also located in periodontal ligament subpopulations and in cells with a paravascular location (Fig. 3B). Alveolar bone and osteoblasts were negative since they did not express amelogenin (Fig. 3B) as was cellular cementum (data not shown). As the human specimens presented root resorption, AMEL expression was localized to the 'cementoid' phase covering the resorption area and cells that could represent putative cementoblasts depositing cementum matrix onto the dentin in the cervical area (Fig. 3G). Ameloblastin expression was found in cells in the vicinity of acellular cementum (Fig. 3D). Very few cells are in intimate contact with cementum and groups of cells representing pre-cementoblasts stained strongly (Fig. 3E). Cells with a paravascular location were also immunopositive for AMBN (Fig. 3E). Cementoblasts located in cellular cementum, cementocytes, alveolar bone and osteoblasts did not express AMBN.

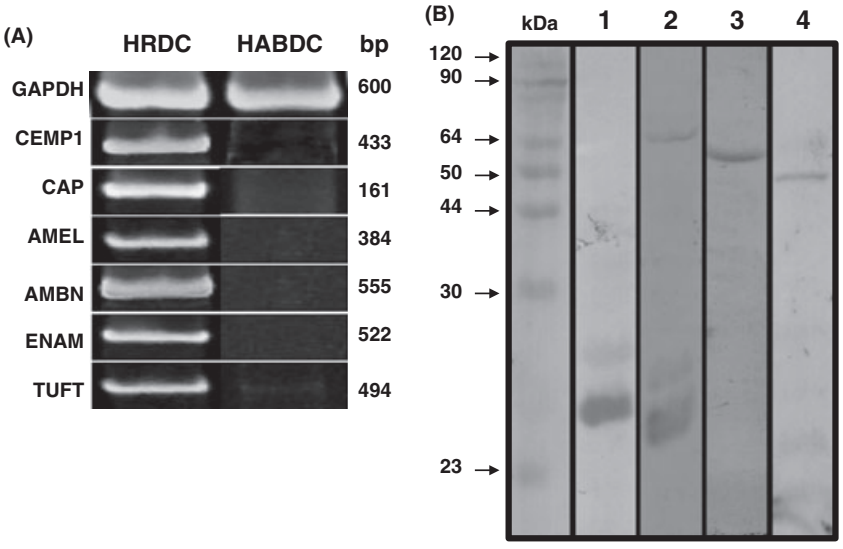

Fig. 1. (A) The RT-PCR amplification of cementum attachment protein (CAP), cementum protein 1 (CEMP1), amelogenin (AMEL), ameloblastin (AMBN), enamelin (ENAM) and tuftelin (TUFT) transcripts in human root-derived cementoblasts (HRDC) and human alveolar bone-derived osteoblasts (HABDC). The use of gene-specific primer sets for RT-PCR amplification generates a 433-bp amplicon for CEMP1, a 161-bp amplicon for CAP, a 384-bp amplicon for AMEL, a 555-bp amplicon for AMBN, a 522-bp amplicon for ENAM and a 494-bp amplicon for TUFT. The HABDC did not express any of the transcripts. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as a control. (B) Western blot analyses indicating the expression of protein species of 27 kDa (AMEL, lane 1), 62 kDa (AMBN, lane 2), 55 kDa (CAP, lane 3) and 50 kDa (CEMP1, lane 4).

Discussion

In the present study we successfully isolated and established a cementoblast cell line derived from normal human root cementum. These cells are truly cementoblasts because they express (at the mRNA and protein levels) the only cementum markers available to date, namely CAP and CEMP1 (8). These molecules have been shown to play an important role during cementoblast differentiation and biomineralization of cementum extracellular matrix (14). Recently, it has been shown that CEMP1 possesses a strong affinity for hydroxyapatite and promotes octacalcium phosphate crystal nucleation (15). These molecules are also expressed by paravascular cells within the periodontal ligament that represent ancestors of cementoblasts, osteoblasts and periodontal ligament fibroblasts. CAP and CEMP1 represent biological markers for cementoblasts and cementoblast-progenitor cells because CEMP1 co-localizes with periodontal ligament stem cells expressing STRO1 (16). Furthermore, human osteoblast cells do not express these proteins,

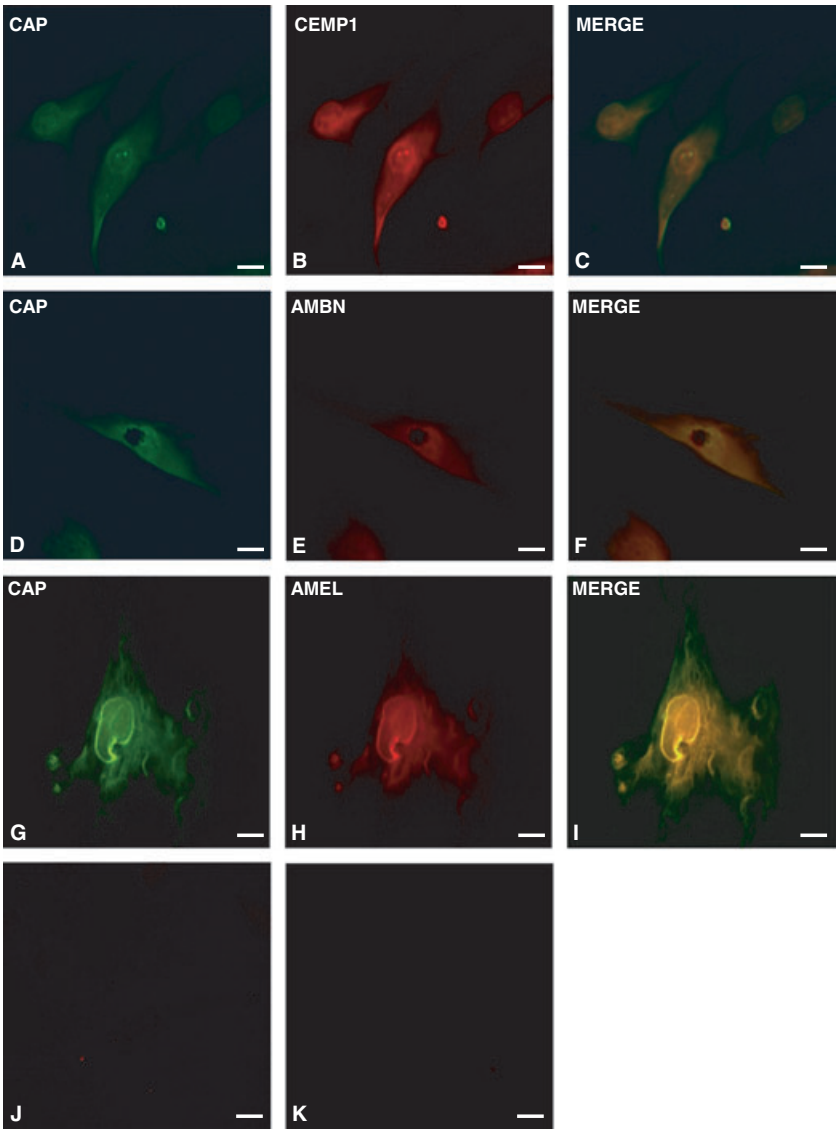

Fig. 2. Double staining and co-localization of cementum attachment protein with cementum protein 1 (CEMP1), amelogenin (AMEL) and ameloblastin (AMBN) in human root-derived cementoblasts (HRDC) and human alveolar bone-derived osteoblasts (HABDC). Cementum attachment protein (CAP) localizes to the cell cytoplasm (A, D and G). CEMP1 localizes to the cell cytoplasm and nucleus (B). Co-localization of CEMP1 and CAP indicates that CAP stains fiber-like cell structures as well as the cytoplasm and the nucleus (C). Ameloblastin stains the cell cytoplasm (E) and co-localizes with CAP at the same cell structures (F). Amelogenin stains the cytoplasm and the nucleus and co-localizes with CAP at the same cell structures (H), except that it does not stain fiber-like structures (I). Representative negative controls using normal rabbit (J) or mouse (K) serum are negative. Magnification: × 40. Scale bar = 100 μm.

which confirms that they have a different cell phenotype. Nevertheless, the expression of CAP, CEMP1 and AMEL in the nucleus of HRDC could be associated with the role of these proteins as 'growth factors' related to proliferation and differentiation processes (11,17).

In previous reports it was shown that antibodies to enamel proteins cross-react with proteins extracted from mouse cementum, suggesting that cementum contains a unique class of enamel-associated or enamel-like proteins (18,19). Furthermore, EMDs have been used to induce *de novo* cementum formation and periodontal ligament repair in both experimental animals and humans (20,21). It was later demonstrated that AMBN is expressed by Hertwig's epithelial root sheath cells (HERS) cells, both *in vivo* and *in vitro*. Our results clearly show that human root cementum-derived cementoblasts express not only AMBN, but also other enamel-associated proteins such as AMEL, ENAM and TUFT. In addition, the expression of AMEL and AMBN by cementoblasts was confirmed in human tissues using immunohistochemistry. To our knowledge, this is the first report that demonstrates the expression of specific enamel-associated molecules by human cementoblasts, *in vitro* and *in vivo*.

Both AMEL and AMBN promote cell attachment and proliferation of periodontal ligament cells *in vitro*. Leucine-rich AMEL peptide has been suggested to act as a signalling molecule capable of inducing the osteoblast phenotype in various types of cells, including mouse embryonic stem cells (22). Consequently, it is possible that these proteins might participate during cementoblast differentiation in the adult periodontium by inducing undifferentiated mesenchymal cells into the cementoblast phenotype. Interestingly, cementoblasts, cementoid phase, mineralized matrix of human cementum, periodontal ligament cell subpopulations and putative stem cells located paravascularly are positive for AMEL. Our results also support the role of AMEL during cementum repair/regeneration, because areas of root resorption and the matrix being deposited by cementoblasts expressed AMEL. This finding is in agreement with other authors reporting that HERS cells produce cementum-related and/or enamel-related proteins (1,3), which play a pivotal role in the differentiation of cementoblasts that specifically produce acellular extrinsic fiber cementum (6,7). Our results show that enamel-related proteins are only expressed by cells associated with the acellular cementum. While the origin of enamel proteins in cementum could be attributed to the stem cells in the periodontal ligament, the exact

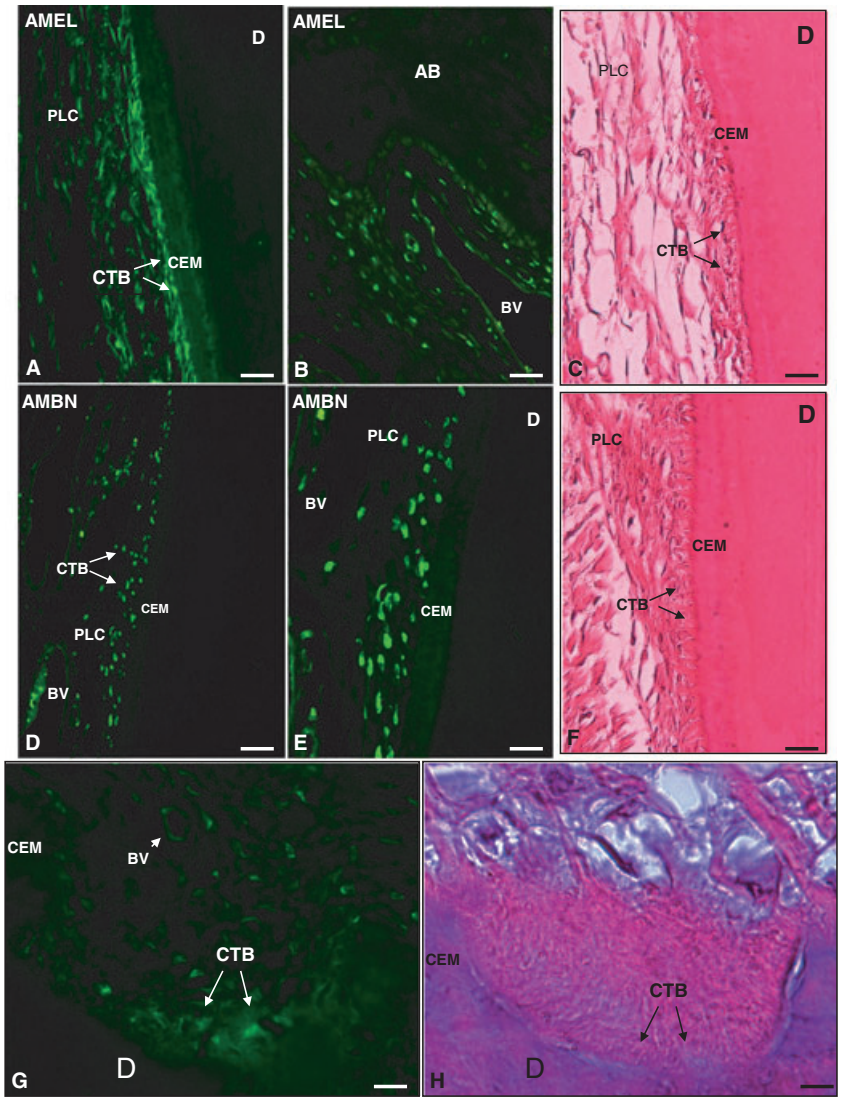

Fig. 3. Immunostaining of human periodontal tissues shows amelogenin staining of cementoblasts, cementoid phase and periodontal ligament cell subpopulations (A). Alveolar bone is negative. Putative stem cells with a paravascular location and periodontal ligament cell subpopulations stain strongly with polyclonal anti-amelogenin (B). Polyclonal anti-ameloblastin cross-reacts with cell clusters in the vicinity of acellular cementum and with cells close to the periodontal ligament blood vessels (D and E). The root resorption area shows that cells and the matrix they are depositing immunoreact with polyclonal anti-amelogenin (G). Histological hematoxylin and eosin-stained sections show the anatomical aspects of experimental slides described above (C, F and H). Magnification in A, C, D and F: × 20 (scale bar = 100 μm). Magnification in B and D: × 40 (scale bar = 50 μm). Magnification in G and H: × 100 (scale bar = 20 μm). AMBN, ameloblastin; AMEL, amelogenin; BV, blood vessel; CEM, cementum; CTB, cementoblast; D, dentin; PLC, periodontal ligament cells.

mechanisms that regulate cementoblast differentiation are not known. Nevertheless, it has been suggested that HERS cells undergo ecto-mesenchymal transformation and acquire morphological and phenotypic changes leading to acellular cementum formation, while neural crest-derived cementoblasts are in charge of synthesizing cellular cementum (23,24).

Taken together, our results suggest that enamel-associated proteins and cementum proteins could act synergistically, regulating cementoblast differentiation and cementum deposition. These data offer new approaches to determine how the cementogenesis process is regulated and point out the role of these proteins during periodontium homeostasis and regeneration of periodontal structures.

Acknowledgements

This study was supported by funds from DGAPA-UNAM (IN200908), CONACyt 48638 and 130950 to H.A.

References

1. Bosshardt DD, Zalzal S, McKee MD, Nanci A. Developmental appearance and distribution of bone sialoprotein and osteopontin in human and rat cementum. *Anat Rec* 1998;**250:**13–33.
2. Hammarström L, Alatli I, Fong CD. Origins of cementum. *Oral Dis* 1996;**2:** 63–69.
3. Narayanan AS, Bartold M. Biochemistry of periodontal connective tissues and their regeneration: a current perspective. *Connect Tissue Res* 1996;**34:**191–201.
4. Booshardt DD. Are cementoblasts a subpopulation of osteoblasts or a unique phenotype?. *J Dent Res* 2005;**84:**390–406.
5. Seo BM, Miura M, Gronthos S *et al.* Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;**364:**149–155.
6. Alvarez-Perez MA, Narayanan S, Zeichner-David M, Carmona-Rodríguez B, Arzate H. Molecular cloning, expression and immunolocalization of a novel human cementum-derived protein (CP-23). *Bone* 2006;**38:**409–419.
7. Hammarström L. Enamel matrix, cementum development and regeneration. *J Clin Periodontol* 1997;**24:**658–668.
8. Hammarström L. The role of enamel matrix proteins in the development of cementum and periodontal tissues. *Ciba Found Symp* 1997;**205:**246–255.
9. Núñez J, Sanz-Blasco S, Vignoletti F *et al.* 17ß-estradiol promotes cementoblast proliferation and cementum formation in experimental periodontitis. *J Clin Periodontol* 2010 (in press).
10. Arzate H, Alvarez-Perez MA, Aguilar-Mendoza ME, Alvarez-Fregoso O. Human cementum tumor cells have different features from human osteoblastic cells *in vitro*. *J Periodontal Res* 1998;**33:**249–258.
11. Carmona-Rodríguez B, Alvarez-Pérez MA, Narayanan AS *et al.* Human cementum protein 1 induces expression of bone and cementum proteins by human gingival fibroblasts. *Biochem Biophys Res Commun* 2007;**358:**763–769.
12. Hosoya A, Nakamura H, Ninomiya T *et al.* Immunohistochemical localization

of alpha-smooth muscle actin during rat molar tooth development. *J Histochem Cytochem* 2006;**54:**1371–1378.
13. Shi SR, Cote C, Kalra KL, Taylor CL, Tandon AK. A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin embedded human temporal bone sections for immunohistochemistry. *J Histochem Cytochem* 1992;**40:** 787–792.
14. Alvarez Pérez MA, Pitaru S, Alvarez Fregoso O, Reyes Gasga J, Arzate H. Anticementoblastoma-derived protein antibody partially inhibits mineralization on a cementoblastic cell line. *J Struct Biol* 2003;**143:**1–13.
15. Villarreal-Ramírez E, Moreno A, Mas-Oliva J *et al*. Characterization of recombinant human cementum protein 1 (hrCEMP1): primary role in biomineralization. *Biochem Biophys Res Commun* 2009;**19:**384.
16. Garcia de Paula e Silva FW, Ghosh A, Arzate H, Kapila S, Bezerra da Silva LA, Kapila Y. Calcium hydroxide promotes cementogenesis and induces cementoblastic differentiation of PDL cells in a CEMP-1 and ERK-dependent manner. *Calcif Tissue Int* 2010 (in press).
17. Zeichner-David M, Chen LS, Hsu Z, Reyna J, Caton J, Bringas P. Amelogenin and ameloblastin show growth-factor like activity in periodontal ligament cells. *Eur J Oral Sci* 2006;**114**(suppl 1)**:**244–253.
18. Slavkin HC, Bessem C, Fincham AG *et al*. Human and mouse cementum proteins immunologically related to enamel proteins. *Biochim Biophys Acta* 1989;**25:** 12–18.
19. Slavkin HC, Bringas P Jr, Bessem C *et al*. Hertwig's epithelial root sheath differentiation and initial cementum and bone formation during long-term organ culture of mouse mandibular first molars using serumless, chemically-defined medium. *J Periodontal Res* 1989;**24:**28–40.
20. Hammarström L, Heul L, Gestrelius S. Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins. *J Clin Periodontol* 1997;**24:**669–677.
21. Heul L. Periodontal regeneration with enamel matrix derivative in one human experimental defect. A case report. *J Clin Periodontol* 1997;**24:**693–696.
22. Warotayanont R, Zhu D, Snead ML, Zhou Y. Leucine-rich amelogenin peptide induces osteogenesis in mouse embryonic stem cells. *Biochem Biophys Res Commun* 2008;**367:**1–6.
23. Thomas HF. Root formation. *Int J Dev Biol* 1995;**39:**231–237.
24. Zeichner-David M, Oishi K, Su Z *et al*. Role of Hertwig's epithelial root sheath cells in tooth root development. *Dev Dyn* 2003;**228:**651–663.