Revista Odontológica Mexicana
Facultad de Odontología
Vol. 17, No. 2 ● April-June 2013
pp 76-80
ORIGINAL RESEARCH

Production and characterization of a cement (CEMP1) recombining protein in *Drosophila melanogaster* (DML-2-23) cells

Producción y caracterización de una proteína recombinante del cemento (CEMP 1) en células de Drosophila melanogaster *(DML-2-23)*

Adriana Rivera Priego,* Bruno Carmona Rodriguez,§ Higinio Arzate§

ABSTRACT

To the present date, molecular and cellular factors which regulate cement formation and mineralization processes are not well known. This is mainly due to the lack of a biological marker for this type of tissue. We have recently isolated, cloned and expressed a protein derived from human radicular (root) cement, called cement protein. This protein is expressed by cementoblasts as well as periodontium progenitor cells. The aim of the present work was to express cement protein in *Drosophila melanogaster* cells, so as to determine, in the future, its post-translational modifications. Our results show we have established a cellular line which expresses protein cement in an essential and stable fashion. This fact is of unique importance, since in the mediate future these cells will become the vehicle for cement protein production in sufficient amounts to determine in a predictable manner, its role during the process of *de novo* periodontium formation process in animal models.

RESUMEN

Los factores celulares y moleculares que regulan los procesos de formación y mineralización del cemento son poco conocidos hasta la fecha. Principalmente esto se debe a que no existe un marcador biológico de este tejido. Nosotros recientemente hemos aislado, clonado y expresado una proteína derivada del cemento radicular humano llamada proteína del cemento. Esta proteína es expresada por cementoblastos y células progenitoras del periodonto. El objetivo de este trabajo fue el de expresar la proteína del cemento en células de *Drosophila melanogaster* para en un futuro, determinar sus modificaciones postraduccionales. Nuestros resultados muestran que hemos establecido una línea celular que expresa constitutivamente y de forma estable la proteína del cemento. Esto es de singular importancia, ya que en un futuro mediato, estas células serán el vehículo para la producción de la proteína del cemento en cantidades suficientes para determinar su papel durante el proceso de la formación del periodonto *de novo* en modelos animales y de un modo predecible.

Key words: Cementum, CEMP1, cementogenesis, periodontal regeneration.
Palabras clave: Cemento, proteína del cemento, cementogénesis, regeneración periodontal.

INTRODUCTION

One of the main problems encountered in periodontal disease is the destruction of tissues providing insertion of teeth into the alveolar bone. Therapeutic strategies directed to solving this problem aim at achieving full regeneration of periodontal tissues.[1] In spite of advances experienced with respect to osteogenesis, the processes which regulate cementogenesis are not well known. For this reason our understanding of cement and periodontium regeneration is still obscure.[2] Substantial advantages in the field of bone, enamel and dentin formation have taken place due to the availability of specific markers which facilitate identification of these tissues.[3] In contrast to this, the lack of specific markers for cement has limited our knowledge on mechanisms that control differentiation of cementoblastic cells, cement formation as well as periodontium development. Several laboratories, ours included among them, after dedicated effort, have achieved isolation of several cement specific putative proteins, such as 55kDa called cement adhesion protein (CAP),[4,5] a protein related and derived from human cementoblast (CP)[6] a mitogenic factor known as Root Cement Growth Factor (CGF) as well as an isoform of growth factor similar to insulin (IGF-I),[7] and a 72kDa protein, CEM-1.[8] Complete characterization

* Student, Orthodontics Specialty, Graduate School National School of Dentistry, National University of Mexico (UNAM).
§ Molecular and Cellular Biology Laboratory, National School of Dentistry, National University of Mexico (UNAM).

of these proteins has been an arduous and even sometimes unsuccessful task. Recently, in our laboratory, we were able to isolate and characterize a gene derived from a DNAc library (complementary DNA called «cement protein CEMP1») which appears to be specific for cells belonging to the cementoblastic cell lineage and periodontal ligament cellular sub-populations.[8] Preliminary data suggest the fact that E coli hrCEMP1 protein bears effect on alkaline phosphatase proliferation and activity in cementoblastic cells. It is then important to determine whether protein bears effect into other cellular functions. Nevertheless, CEMP1 primary structure analysis using PROSITE database revealed that this protein can be post-transcriptionally modified. To determine these modifications it is necessary to use *Drosophila* cellular expression system (DES Invitrogen, CA). This system offers an advantage over other mammal's expression systems: it can produce large amounts of recombining protein. Therefore, the main objective of this project is to express this gene in a eukaryotic cellular system in embryonic cells of *Drosophila Melanogaster* DML-2. This had the purpose of achieving sufficient amounts of this protein to determine its qualities with respect to its biological activity.

MATERIALS AND METHODS

CEMP1 GENE TRANSFECTION INTO DML-2 CELLS

The expression system in *Drosophila* cells that was used was acquired from Invitrogen (Carlsbad, CA). Manufacturer's procedures were closely adhered to. Briefly, the cDNA of CEMP1 protein was cloned in pMT/BiP/V5/-His plasmid. This resulted in pMT/BiP/CEMP1-His plasmid. In it, the terminal-N terminus of hCEMP1 protein is fused with the BiP signal sequence, while in its terminal C terminus it fuses to a peptide (histidine) 6 X (construction was confirmed through sequencing). CEMP1 protein transcription is found under regulation of the metallothionein promoter (MT). To transfect, *Drosophila melanogaster* embryonic S2 cells were used and plasmid lacking CEMP1 protein codifying sequence was used as negative control. Transfected cells were used to express CEMP1 through the addition of $CuSO_4$. S2 cells and their culture medium were tested for CEMP1 expression with the help of western blot using an antibody anti-His and anti- hrCEMP1. After confirming that CEMP1 was adequate, S2 cells were co-transfected with vector pMT/BiP/CEMP1-His and selection vector pCoHygro. Cells grew in DES complete expression medium with hygromycin B for its selection and stable expression during 8 weeks. Selection medium was replaced every 5 days so as to select resistant cells which were established as stable cellular line. These cells were introduced with 500 μM of $CuSO_4$/mL. Cells and medium were assessed and cellular lines with highest CEMP1 yield were selected. Recombining protein was purified by passing the medium through a Ni-NTA agarose column. Purity was determined with the aid of a polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE), silver tincture and Western blot.

CEMP1 PROTEIN (CEMENT PROTEIN) IMMUNOLOCATION IN *DROSOPHILA DML-2* CELLS

In order to determine whether cells transfected with CEMP1 gene expressed this protein, immune-location trials of these cells were undertaken. These trials were as follows: DML-2 cells were planted at low density (5 x 10^2) in eight-well, Lab-tek sterile boxes. Cells were left to adhere overnight and were cultivated for three days. Cells were fixated in 3.7% formaldehyde and immunolocation procedures were undertaken. Number of cells reactive to anti-hrCEMP1 were determined by quantifying five different fields under a microscope with 20 X lens. Results are expressed as averages (n = 5) and standard deviation of three independent experiments. Cultures were incubated in pre-immune rabbit serum, or without the first antibody, to be used as negative controls.

IMMUNOBLOT

Western blots were performed to determine whether DLM-2 cells transfected with CEMP1 gene expressed this marker. For this aim, monoclonal (Anti-His-6X C-term, Invitrogen, Carlsbad CA) antibodies were used. Policlonal anti hrCEMP1 antibodies were also used. They were used to identify recombining protein histidines and specifically recognize CEMP1 protein respectively. To test each antibody, equal amounts of protein (5 μg/well) were loaded into a 12% SDS-PAGE gel following standard procedures. HrCEMP1-DML2 was transferred to an Immobilon-P (PVDF) (Millipore Corp Bedford MA) nitrocellulose membrane. Membranes were blocked during one hour with a buffer (shock absorber) containing 100 ml M Tris-HCl pH 7.5 and 5% skimmed milk. At a later point, membranes were incubated with anti-hrCEMP1

(1:300) and anti-His monoclonal antibody (1:5,000). Membranes were incubated for one hour at room temperature. After being washed, membranes were incubated in a secondary antibody conjugate anti-rabbit or anti-mouse, conjugated to radish peroxidase (1:1,000) during one hour. Membranes were washed and revealed as previously described.

RESULTS

CEMP1 GENE TRANSFECTION INTO DML-2 CELLS

Once transfected, DML-2 cells were subjected to a transitory expression of CEMP1 protein. They were incubated in the presence of $CuSO_4$, 500 μM of CuSO4/mL during 48 hours. After this time, both cells and conditioning medium were collected and processed for SDS-PAGE and immunoblot in the manner previously described. In order to obtain a cellular line with CEMP1 protein cellular expression, these cells were selected with hygromycin B (600 μg/mL, during 8 weeks), and protein expression was determined as was explained before in this paper. HrCEMP1 protein immune-location as well as successful transfection of CEMP1 gene were determined with the help of a monoclonal antibody anti-His-COOH-conjugated to FITC. Our results revealed the fact that CEMP1 protein was located in the cell membrane *(Figures 1A and 1B)* respectively. Controls conducted with pre-immune rabbit and mouse serum did not show evident reaction *(Figures 1C and 1 D respectively)*. In order to determine CEMP1 protein expression in DMEL2 cells, immunoblots were performed using antibodies against protein rCEMP1 and anti-His-COOH. Conditioning medium and cellular extract were collected. Our results showed that when dyeing with Coomassie blue, the most prominent band was observed to have 55-60 kDa approximate molecular weight in the conditioning medium *(Figure 2, line 1)*. Likewise, immunoblots with the aforementioned antibodies revealed they recognized species of approximately 55-60 kDa *(Figure 2, lines 3 and 5)*.

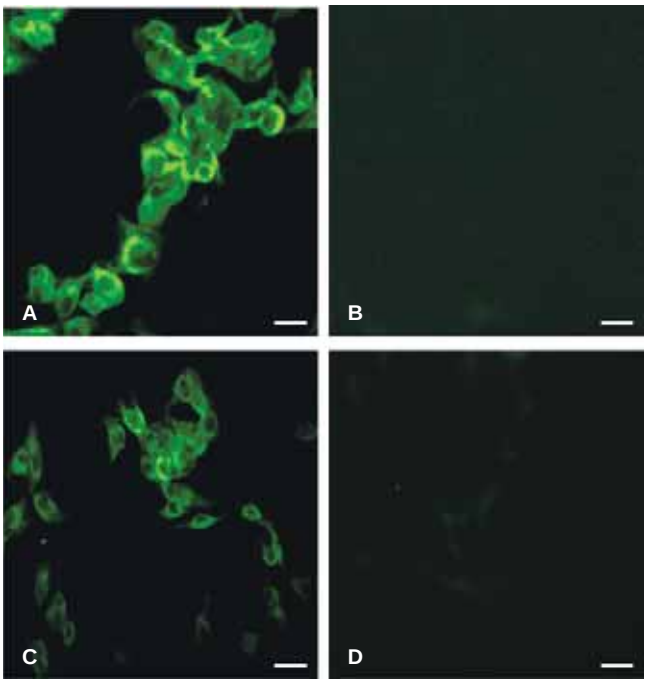

Figure 1. A) It is observed that cells transfected with cement protein gene are recognized by anti 6X his monoclonal antibody. In figure **C)** we can observe cement protein expression using a recombining antibody against this. In figures **B)** and **D)** we observe lack of positive recognition of this protein. They correspond to negative controls. Bar = 100 μm.

DISCUSSION

In this research project, a description was made of the construction and production of a human recombining protein derived from cement (CEMP1) in *Drosophila melanogaster* cells. Cement specific proteins such as CAP and CP have previously been described, as well as having recently being cloned, characterizing and expressing this latter's gene product, which we have named «cement protein» (CEMP1).[9] Preliminary studies suggest that hrCEMP 1 protein in *E. coli* bears effect upon cellular chemotaxis and proliferation and promotes alkaline phosphatase activity in cells derived from in vitro human periodontium.[4,10] Nevertheless, and considering the fact that expression's prokaryotic systems such as *E. coli* do not possess the means to perform post-translational modifications of gene products, and aiming at knowing these, we propose the possibility of using an eukaryotic expression system using embryonic cells derived from *D melanogaster* flies. In the present research paper we report expression of cement protein (CEMP1) in the aforementioned cells. Their stable expression will enable, in the future, to determine post-translational modifications of this protein, such as phosphorylations and glycosylations.

Production of the protein was substantiated in SDS-PAGE gels dyed with Coomassie blue, as well as Western blot using specific antibodies against CEMP1 protein and antibody against 6x histidines. Our results revealed that cement protein (CEMP1)

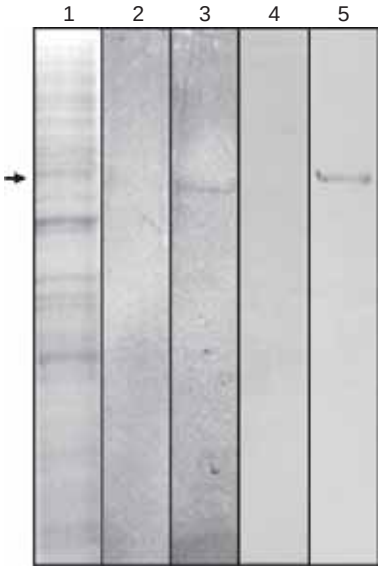

Figure 2. Line 1 shows protein dyeing with Coomassie blue expressed by *Drosophila melanogaster* cells and transfected with the cement protein gene. The arrow indicates 50 kDa species. Line 2 represents negative control of cells which do not constitutively express the cement protein gene. Line 3 shows recognition of a 50 kDa band by means of anti-6Xhis antibody. Line 4 represents negative control using recombining antibody against cement protein. Line 5 shows a 50kDa band recognized by the cement anti-protein antibody.

has a M_r of 50,000, as was shown through the use of Western blots. Likewise, our results determined that this protein is located in the cellular membrane of these cells, and is also secreted into the conditioning medium.[11] These preliminary findings offer us the possibility of determining whether post-translational modifications of CEMP1 protein could affect its biological activity. Therefore, essentially, availability of this gene will allow us to determine cellular and molecular events which regulate the cementogenesis process during developing and adult periodontium as well as in regenerative processes. Even more, it could promote new ways for the design of pre-clinical studies aimed at achieving cementogenesis *de novo* and regeneration of periodontal tissues in a predictable manner. As has been previously demonstrated, this protein seems to be associated to the mineralization process, since it regulates composition and morphology of hydroxyapatite crystals.[12] Likewise, it regulates expression of the alkaline phosphatase, a factor associated to early stages of mineralization processes. Bone sialoprotein is associated to the nucleation process of hydroxyapatite crystals and to their growth control.[12] In a similar manner, cement protein regulates as well the osteopontin expression, which is a protein associated to the regulation of the mineralization process, mainly regulating the growth of hydroxyapatite crystals in the late stages of the mineralization process.

CONCLUSIONS

Based on results obtained in this research paper, we beg to propose that cement protein could play an important regulating role during cementogenesis process. Of even greater importance are the possibilities offered by the biological characteristics of this new protein insofar as predictable treatment to promote regeneration of tissues conforming the periodontium.[13] This innovative protein opens as well new ways to implement therapies designed for regeneration of mineralized tissues.

REFERENCES

1. Ripamonti U, Reddi AH. Periodontal regeneration: potential role of bone morphogenetic proteins. *J Periodont Res* 1994; 29 (4): 225-235.
2. MacNeil RL, D'Errico JA, Ouyang H, Berry J, Strayhorn C, Somerman MJ. Isolation of murine cementoblasts: unique cells or uniquely-positioned osteoblasts? *Eur J Oral Sci* 1998; 106 (Suppl 1): 350-356.
3. Paynter KJ, Pudy G. A study of the structure chemical nature, and development of cementum in the rat. *Anat Rec* 1958; 131: 233-251.
4. McAllister B, Narayanan AS, Miki Y, Page RC. Isolation of a fibroblast attachment protein from cementum. *J Periodont Res* 1990; 25 (2): 99-105.
5. Arzate H, Chimal-Monroy J, Hernández-Lagunas L, Díaz de León L. Human cementum protein extract promotes chondrogenesis and mineralization in mesenchymal cells. *J Periodont Res* 1996; 31(2): 144-148.
6. Arzate H, Jiménez-García LF, Álvarez-Pérez MA, Landa A, Bar-Kana I, Pitaru S. Immunolocalization of a human cementoblastoma conditioned medium-derived protein. *J Dent Res* 2002; 81 (8): 541-546.
7. Nakae H, Narayanan, AS, Raines E, Page RC. Isolation and partial characterization of mitogenic factor from cementum. *Biochemistry* 1991; 30 (29): 7047-7052.
8. Slavkin HC, Bringas PJr, Bessem C, Santos V, Nakamura M, Hsu My 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 Periodont Res* 1989; 24 (1): 28-40.
9. Alvarez-Pérez MA, Narayanan AS, Zeichner-David M, Rodríguez Carmona B, Arzate H. Molecular cloning, expression and immunolocalization of a novel human cementum-derived protein. *Bone* 2006; 38 (3): 409-419.
10. Zeichner-David M. Regeneration of periodontal tissues: cementogenesis revisited. *Periodontol 2000* 2006; 41: 196-217.
11. Carmona-Rodríguez B, Álvarez-Pérez MA, Narayanan AS, Zeichner-David M, Reyes-Gasga J, Molina-Guarneros J et al.

Human cementum protein 1 (CP-23) induces cell differentiation and mineralization in human gingival fibroblasts in vitro. *Biochemical and Biophysical Research Communications*; 358 (2007): 763–769.

12. Álvarez Pérez MA, Pitaru S, Álvarez Fregoso O, Reyes Gasga J, Arzate H. Anti- cementoblastoma-derived protein antibody partially inhibits mineralization on a cementoblastic cell line. *J Struct Biol* 2003; 143(1): 1-13.
13. 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.

Mailing address:
Higinio Arzate
E-mail: harzate@unam.mx