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

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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 its role during the process of de novo periodontium formation process in animal models.

Key words: Cementum, CEMP1, cementogenesis, periodontal regeneration.

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 of these proteins not only can facilitate the identification of these tissues, but also can determine their role during the processes that lead to cement formation.9 Thus far, no cement specific marker has been identified which can distinguish between differentiating cementoblasts and periodontium progenitor cells, thus making it difficult to isolate pure populations of cementoblasts, a problem that is crucial for the establishment of new strategies aimed at regenerating this tissue.

One of the main advantages of Drosophila melanogaster has been its usefulness as an animal model for studying developmental processes due to its large number of genes and the ease and speed with which certain developmental processes can be studied.10 As an early representative of the metazoan phylum, D. melanogaster is well suited to address fundamental questions on the regulation of cell differentiation and development.11 Thus far, the study of cement formation in D. melanogaster has been limited due to the lack of available markers to distinguish between differentiating cementoblasts and periodontium progenitor cells. Therefore, to be able to identify and study cement formation in this model organism, it is crucial to establish cement specific markers which can allow for the isolation of pure populations of cementoblasts. The isolation of a cement specific marker in D. melanogaster would allow for the study of bone development in this model organism, which could provide new insights into the processes that lead to cement formation in the mammalian system.9

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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. 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 recombinant 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 CuSO₄. 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 CuSO₄/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, immunolocation trials of these cells were undertaken. These trials were as follows: DLM-2 cells were planted at low density (5 x 10⁵) 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. Polyclonal anti hrCEMP1 antibodies were also used. They were used to identify recombinant 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.
Production and characterization of a cement
(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 $\mu$M 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 $\mu$g/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 1D 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).

DISCUSSION

In this research project, a description was made of the construction and production of a human recombinant 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). Preliminary studies suggest that hrCEMP1 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)
has a M 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


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