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Full Length Research Paper

Recombinant matrix extracellular phosphoglycoprotein (MEPE) can promote mineralization in *vitro*

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The matrix extracellular phosphoglycoprotein (MEPE) gene is highly expressed in tumors that cause oncogenic hypophosphatemic osteomalacia (OHO). MEPE is also known as one of the bone-tooth matrix proteins and is associated with bone and teeth mineralization. We developed a rabbit polyclonal antibody directed against recombinant human MEPE after cloning its cDNA from the cDNA library of a human brain cDNA library. Using this anti-body, we analyzed the distribution of MEPE in dog dental germ tissue by immunohistochemistry. In these specimens, MEPE was predominantly expressed by odontoblast cells and predentin, but not by dental pulp cells. Furthermore we used von kossa staining. And the results suggested that MEPE could induce mineralization and we propose that this protein has a potential effect on dental rehabilitation.

Key words: Matrix extracellular phosphoglycoprotein, mineralization Von kossa.

INTRODUCTION

Recently, a new bone matrix protein cDNA has been cloned from human, rat, and mouse by independent groups (Rowe et al., 2000; Petersen et al., 2000; Argiro et al., 2001). The human clone, termed matrix extracellular phosphoglycoprotein (MEPE), was isolated from a human oncogenic hypophosphatemic osteomalacia (OHO) tumor cDNA library (Rowe et al., 2000). MEPE, bone sialoprotein (BSP), osteopontin (OPN), but sometimes known as SPP1 and Eta-1, dentin matrix protein I (DMP I), and dentin sialophosphoprotein (DSPP) are the products of five genes clustered along human chromosome 4q21 (Rowe et al., 2000; Petersen et al., 2000; Argiro et al., 2001) between EST markers D4S2785 (WI-6336) and D4S2844. The majority of each protein is encoded by the last one or two exons and contains the integrin- binding RGD tripeptide. Another similarity of all these genes is that all introns always interrupt between codons (type 0), thus leaving open the possibility of splicing any two exons together without causing frame shifts. We have named this protein family the small integrin-binding ligand N- linked glycoprotein (SIBLING) family. And it is based not on current theories of their functions (which are poorly understood), but on the simple biochemical and genetic features shared by all members. The human MEPE has 1989 bp cDNA clone and encodes a predicted 525-amino-acid protein rich in Asp, Ser, and Glu residues (26%) containing a 17-amino-acid signal peptide. MEPE contains two N-glycosylation motifs (NNST and NNSR), a glycosaminoglycan attachment site (SGDG), an RGD cell attachment motif, several predicted phosphorylation motifs, and N-myristoylation sites (Rowe et al., 2000).

In dental tissue, MEPE is expressed in odontoblasts during odontogenesis (MacDougall et al., 2002). Previous *in vitro* studies have suggested a correlation between MEPE expression and bone mineralization. Petersen et al. (2000) showed that MEPE mRNA was expressed by fully differentiated osteoblasts and that its expression increased markedly during osteoblast-mediated mineralization of the bone matrix. Argiro et al. (2001) reported a correlation between MEPE expression and bone mineralization after the addition of glycerophosphate to osteoblast culture medium. MEPE

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plays a significant role in osteoblast-mediated mineralization. Liu et al. (2004) has found that Dentonin(a 23-amino-acid peptide derived from MEPE) could promote DPSC proliferation, taking a potential role in pulp repair. That study suggestted that Dentonin affects primarily the innitial cascade of events leading to pulp healing.

In this study, we have cloned MEPE cDNA by real time polymerase chain reaction (RT- PCR), expressed MEPE in *Escherichia coli* cells, purified the recombinant protein, and prepared anti-MEPE antibodies. Then western blot and immunohistochemistry were applied in the study. Finally, Von kossa staining was used to investigate the mineralization ability of MEPE.

MATERIALS AND METHODS

MEPE cDNA clone isolation and amplifications

Human MEPE cDNA sequence from human brain cDNA library (BGI LifeTech Co., Ltd.) was amplified using reverse transcriptase polymerase chain reaction (PCR). The cDNA clones were isolated in 50 µl reaction mixture using human MEPE primers(1 µl, 1 µmol/L, Forward: 5'- CGGAATTCACCAGAGATTCTCAAAGATGCGAG -3'with an EcoR restriction site and 1 µl, 1 µmol/L, Reverse: 5'-CGAAGCTTGAACTCCTGGTGGACTAGTCACC -3' with an Hind III restriction site), high fidelity platinum Pfx DNA polymerase (0.5 µl, 1.5 U, Promega), 10 x PCR Buffer(5 µl), $MgCl_2$(1.5 µl, 50 mmol/L), dNTP Mix(1 µl, 10 mmol/L, Promega), human brain cDNA library (5 µl, denatured 99°C,10 min), and DEPC-treated water (35 µl). PCR amplifications were carried out at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 90 s, and 72°C for 10 min for final extension and held at 4°C. Reaction products were analyzed by electrophoresis of 10 µl samples in 1%(w/v) agarose gels and visualized by ethidium bromide staining under UV light. DL-2000 DNA ladder (TaKaRa, Japan) served as size marker.

Sequencing and sequence analysis

The PCR products were subcloned into vector pGEM®-T and then transformed into *E. coli* DH5 . MEPE DNA was isolated by the Sanger dideoxynucleotide chain termination procedure (Sanger et al., 1977) using ClearCut Miniprep Kit (Stratagene, CA) . Sequences were read by BGI corp (Takara corp, Japan). Each sequencing reaction was performed twice in both directions. Sequencing of PCR fragments confirmed the sequence obtained. Sequence alignments were performed using the computer program BLAST (Altschul et al., 1990; Zhang et al., 1997). Protein sequence analysis (PSA) were performed using the PROSITE (Hofmann et al., 1999) programs.

Expression and purification of MEPE proteins

The PCR products were subcloned into vector pGEM®-T and pGEX-4T-2 and the construct transformed into *E. coli* BL21 (DE3). White clones were then selected on ampicillin plates. The MEPE clones were incubated at 37°C over night, then incubated for a further 3 h at 42°C using IPTG. Recombinant MEPE was purified using calmodulin affinity chromatography resin as described in the Startagene affinity cloning and protein purification kit.

SDS-page

Samples were mixed up with the buffer (0.1% bromophenol blue, 20% glycerin, 2% -mercaptoethanol, 0.1 mol/L Tris- HCl, pH 8.8 and 4% SDS) and denatured at 100°C for 5 min. After centrifugation at 12000 r/min for 10 min, electrophoresis was performed in 1% (w/v) agarose gels. Finally the products were stained with Coomassie Blue (25% isopropanol, 0.25% Coomassie Blue R 250, 5% glacial acetic acid), and bands were visualized by dynamic integrated exposure using an EagleEye II imaging system (Stratagene Corp., La Jolla, CA).

Antibody generation and western blot analysis

High titer polyclonal antiserum was generated by immunization of rabbits with *E. coli* expressed full-length MEPE. MEPE antibody was affinity-purified by chromatography. MEPE-coupled agarose beads by standard methods (Harlow et al., 1988). Protein samples were dissolved in SDS gel-loading buffer (Invitrogen Carlsbad, CA, USA). Separated proteins were transferred to a nitrocellulose membrane (0.45 lm, Bio-Rad, Chicago, IL USA) at room temperature using Semi-dry blotting system (Millipore, Chicago, IL USA). Immunoblotting was performed using anti-MEPE antisera diluted 1:2000 in TBS and 1% bovine albumin (BSA) for 1 h at room temperature. The blots were washed in TBS for 60 min and incubated with anti-rabbit IgG conjugated with HRP (diluted to 1:5000) at room temperature for 60 min. After washing in TBS, immunoreactivity was detected by ECL detection kit (Amersham Pharmacia Biotech., UK).

Immunohistochemical staining

A mandible specimen from young dog was fixed in 4% paraformaldehyde and decalcified in 10% EDTA for 1 h. Bones were embedded into paraffin, and 5 mm sections were placed onto Digene silanated slides (Beltsville, MD) and heated overnight at 50°C. Slides were rehydrated, and endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 in methanol for 30 min at room temperature, and then the sections were incubated in 10% normal goat serum to minimize nonspecific background staining. Sections were blocked in 1% BSA/PBS and incubated overnight at 4°C in a humidified chamber with the rabbit polyclonal antibody diluted at 1:500 in 0.1% BSA/PBS and the rabbit IgG (Vector Laboratory Inc., Burlingame CA) as a control. Secondary anti-body consisting of anti-rabbit IgG Fab fragments linked to horseradish peroxidase (Amersham Pharmacia Biotech) was diluted at 1:100 in 0.1% BSA/PBS and placed onto sections for 1 h at room temperature. Detection was performed using a diaminobenzidene substrate chromagen system according to the manufacturer's instructions (vector laboratory).

Cell culture

Human pulp cells were grown from pulp tissue as reported previously. Normal human pulp was obtained from extracted impacted third molars. These molars were collected from adults (17 to 25 years old) at stomatology hospital of Fourth Military Medical University with patient's informed consent. The whole procedure followed an informed protocol approved by the local ethics committee. After extraction, the teeth were washed with saline and cut at the apex, followed by removal of pulp tissue. The isolated pulp tissues were minced into 1 mm^3 pieces and incubated in minimal essential medium (MEM) (Life Technologies, Inc. Grand Island, N.Y.) supplemented with an antibiotic solution (100 U/ml penicillin-G and 200 ug/ml streptomycin, 5 ug/ml amphotericin B)

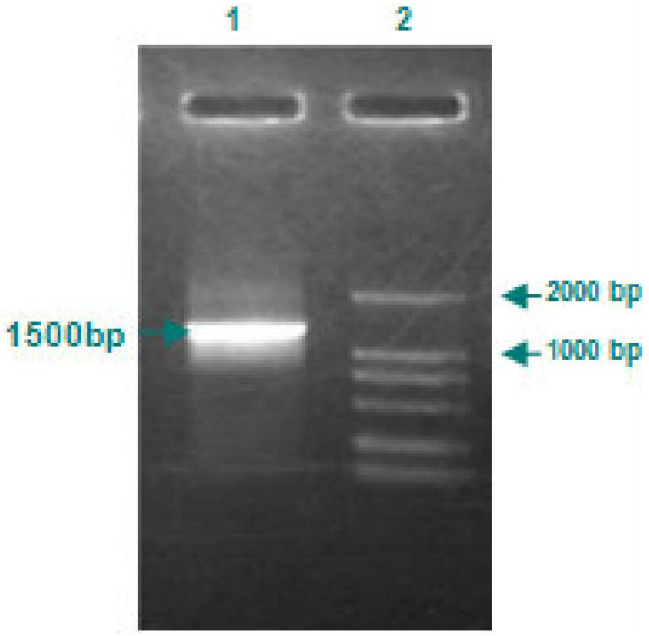

Figure 1. Human MEPE fragment was generated by RT- PCR from human brain cDNA library: A (lane 1: PCR products, lane 2: DL2000) about 1.5 kb was generated by RT-PCR.

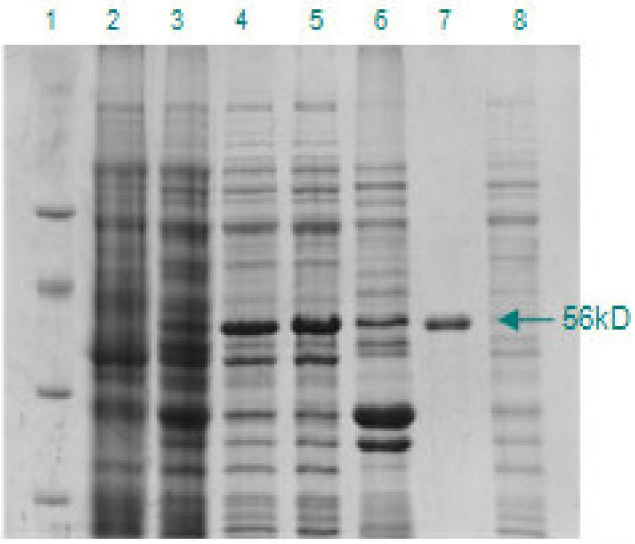

Figure 2. The fusion construct generated by IPTG induction of the *E. coli* host BL21 (DE3) was achieved. The fusion protein was about 56 kD. This is in approximate agreement with the expected molecular size. Purification of recombinant protein was achieved by calmodulin affinity chromatography as described under materials and Methods. (Lane 1: LMW-SDS Marker Kit, lane 2: blank vector, lane 3: non-induced *E.coli*, lane 4: induced *E.coli*, lane 5: lysate supernatant, lane 6: precipitation, lane 7: purified protein, lane 8: culture solution).

and 10% fetal bovine serum (FBS). Cells were grown at standard cell culture conditions (37C, 100% humidity, 95% air and 5% carban dioxide) for 20 days, and the medium was exchanged at 3-day intervals. Cells that were released from the minced pulp explants were harvested and further cultured to acquire sixth-passage cells which were used in this study.

Von Kossa staining

Von Kossa staining was used to evaluate the formation of mineralized nodules on day 15 in 100 µg/ml.Cell cultures were rinsed with PBS and fixed in 4% formaldehyde in PBS for 10 min. After washing with distilled water, cells were treated with 5% silver nitrate solution and kept for 30 min in dark room. The excess silver nitrate solution was then completely washed away using distilled water, and the culture plate was treated with sodium carbonate/formaldehyde solution for few minutes to develop color. Residual silver nitrate was neutralized with 5% sodium thiosulfate. Plates were finally rinsed extensively with water and air-dried. Staining results were captured by canon EOS 550D digital camera.

RESULTS

Isolation and recombinant of MEPE

In this study a human MEPE fragment of about 1.5 kb was generated by RT-PCR from human brian cDNA library (Figure 1) . Identity of the PCR product with the published human MEPE cDNA sequence was confirmed by sequencing. A 1578-bp sequence was obtained. This sequence contained a TAG termination codon. No poly (A) tail or translation initiation codon was detected. The ATG codon at position 40 was selected as the translation initiation codon because it lies in the context of a Kozak criteria (Kozak et al., 1996). Gene content mapping was then performed using the isolated human MEPE/OF45 BAC genomic clone. The predicted secreted MEPE protein has a calculated molecular mass of 56 kDa.

Expression of MEPE proteins and SDS-page

The cDNA coding sequence comprising amino acids 95–525 was subcloned into pGEX-4T-2 as described under materials and methods. Validation of the fusion construct generated by IPTG induction of the *E. coli* host BL21 (DE3) was achieved by SDS-page. The fusion protein was 56 kD (Figure 2). This is in approximate agreement with the expected molecular size. Purification of recombinant protein was achieved by calmodulin affinity chromatography as described under materials and methods.

Western blot analysis and immunohistochemical staining

The specificity of the anti-MEPE antibody was examined by the Western blotting of *E.coli* expressing MEPE. When the *E. coli* lysate was tested, MEPE immunoreactivity was visualized as a single band at 56 kDa (Figure 3A),

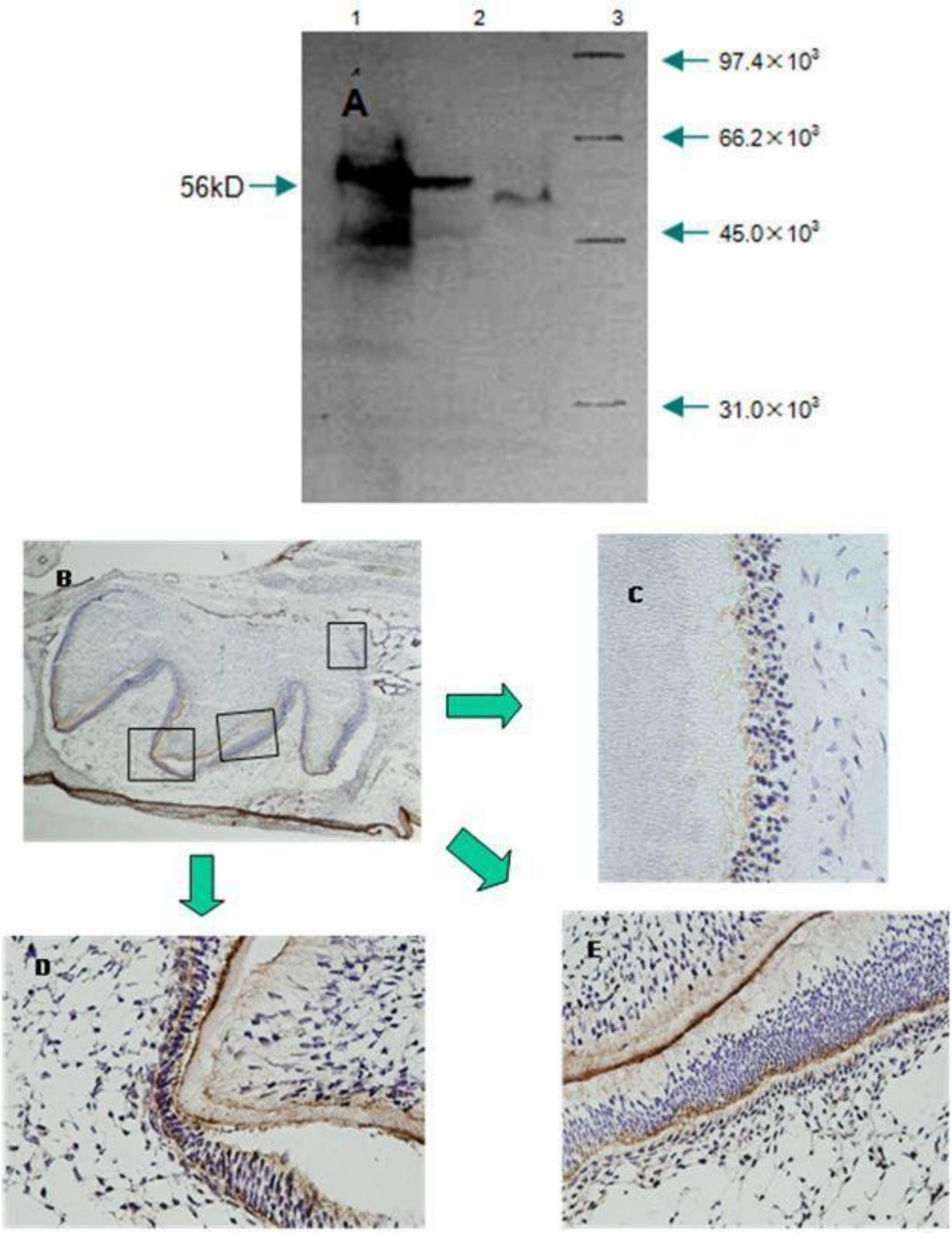

Figure 3. The specificity of the anti-MEPE antibody was examined by the Western blotting of *E.coli* expressing MEPE: (A) (lane 1, 2: induced *E. coli*, lane 3: culture solution, lane 4: LMW-SDS Marker Kit). The fusion protein was about 56 kD in lane 1, 2. This is in approximate agreement with the expected molecular size. (B) Immunohistochemical staining shows MEPE expression in dog grem tissure. (C) MEPE was not expressed in dental pulp cells. (D and E) MEPE was expressed in odontoblasts cell but it was not expressed in dental pulp cells.

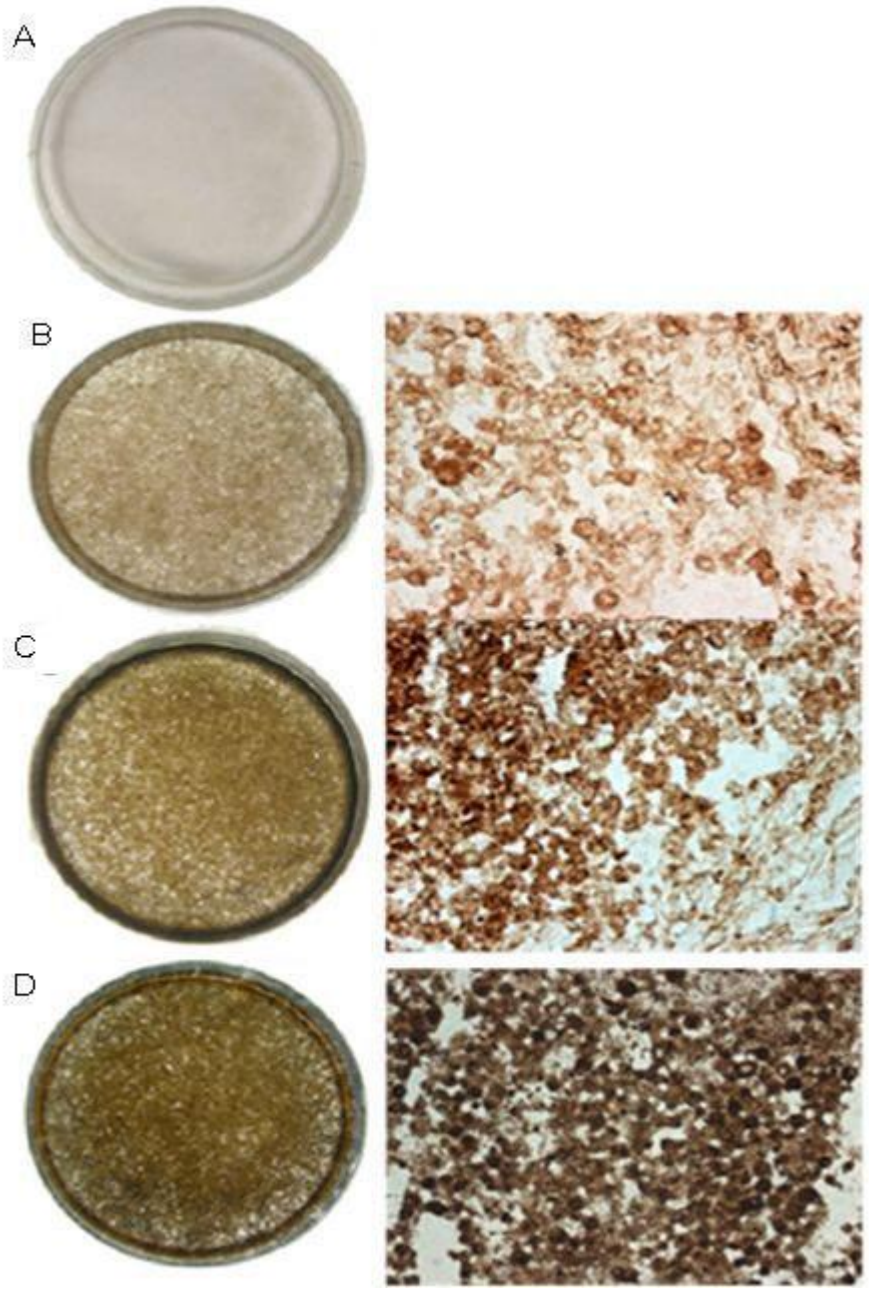

Figure 4. Von kossa staining. MEPE induced HDPCs as assayed using the Von Kossa stain after 28 days. Von Kossa-positive staining was apparent in the control cells with MEPE.(A) 0 day, (B) 7 day, (C) 14 day, (D) 28 day.

demonstrating high specificity of the anti-MEPE antibody. Figures 3B, C and D shows MEPE expression in dog dental germ tissue. MEPE was expressed by odontoblasts cell and predentin, but it was not expressed in dental pulp cells (Figure 3B).

Von Kossa staining

MEPE (100 µg/ml) induced HDPCs as assayed using the Von Kossa stain after 28 days. Von Kossa-positive staining was apparent in the control cells with MEPE. The results for 0, 7, 14, 28 days shown in Figure 4 show quantification of nodule formation after days 7.

DISCUSSION

MEPE is a protein of the extracellular matrix that was first described in tumor-induced osteomalacia where it is

highly expressed and has recently been characterized as a novel bone metabolism regulator. The genomic localization of MEPE on human chromosome 4q21 was refined in relationship to the previously identified dentin/bone gene cluster by gene content mapping. This data suggested that the MEPE gene was potentially located closer to other family members BSP or SPP1 (Argiro et al., 2001). Complete MEPE cDNA encodes 525 amino acid residues and 1989 bp of nucleicacid sequence. The predicted secreted MEPE protein has a calculated molecular mass of 56 kDa. As reported for human MEPE, a specific feature of the MEPE protein is the occurrence in the C-terminus of a serine-rich sequence, DDSSESSDSGSSSES (residues 417–430), that displays homology to repeat motifs found in dentin phosphoprotein, DPP/DSPP(SDSSDSSDSSSSSDSS), DMP1 (SSRRRDDSSESSDSGSSSESDG), osteopontin (DDSHQSDESHHSDESD) and the parent protein, dentin sialophosphoprotein,.

However, its function is still poorly understood (Rowe et al., 2000; Argiro et al., 2001; MacDougall et al., 2002; Gowen et al., 2003; Guo et al., 2002; Quarles et al., 2003). To better understand the role of MEPE in dental formation, repair and remodeling, we isolated and characterized the isolation of a cDNA encoding the murine homologue of human MEPE and demonstrated its expression in *E. coli*. Then we purified the recombinant protein and studied the effects of MEPE on HA through Von kossa staining.

In this study, a human MEPE mRNA species of 1.5 kb was identified from human brain cDNA library (MacDougall et al., 2002) which was consistent with the reported expression of human MEPE (Rowe et al., 2000). Previous *in vitro* studies have suggested a correlation between MEPE expression and bone mineralization. Argiro et al. (2001) reported a correlation between MEPE expression and bone mineralization after the addition of glycerophosphate to osteoblast culture medium. MEPE plays a significant role in osteoblast-mediated mineralization. These dentin-specific proteins are expressed by fully differentiated odontoblasts prior to the onset of mineralization (D'Souza et al., 1992; Bronckers et al., 1993) and are believed to play a role in dentin mineralization ((Butler et al., 1997). We used an immunohistochemical approach and found that MEPE protein was expressed in dog dental germ tissures especially in odontoblasts cell and predentin. These results indicated that during dentin development, MEPE might be involved in the initiation mineralization. Therefore, Mepe could play a role during the late stage of bone growth and remodeling. But the expression of MEPE by odontoblasts is not surprising, since all the other SIBLING members are expressed by both odontoblasts and osteoblasts. Bone and dentin are both collagenous mineralized extracellular matrices that consist of similar ECM proteins.

The process of formation of mineralized nodules has three main developmental stages: proliferation, development of the extracellular matrix and mineralization (Chen et al., 1956). Some studies with a variety of different experimental systems have attempted to determine the effects of mineralized tissue proteins *in vitro*. The most exhaustively studied of these proteins is DPP. DPP has been shown to inhibit the mineralization in solution (Doi et al., 1992) and agarose gels (Fujisawa et al., 1987), and the growth of HA seed crystals (Termine et al., 1980) and the BSP, but not osteopontin, is capable of inducing the mineralization. Several workers have found growth of mineralization *in vitro* ((Fujisawa et al., 1987; Termine et al., 1980; Nawrot et al., 1976; Boskey et al., 1990). These investigations have yielded contradictory findings, with phosphophoryn promoting mineralization formation in some systems and inhibiting it in others. It does seem clear that phosphophoryn covalently attached to agarose beads acts as a potent nucleator of mineralization formation in solutions of low calcium phosphate supersaturation (Linde et al., 1989). No studies on the effect of MEPE on mineralization formation have been reported. Our experiment confirm that MEPE has strong mineralization ability on Von Kossa assay. The activities of MEPE suggest that is most likely to be involved in the initiation of mineralization in dentine respectively. It is possible that MEPE may effectively stimulate DPSC proliferation *in vivo*, and consequently enhance the ability of injured pulp to survive trauma such as occurs in dental restorations. Further studies to determine the role of MEPE in odontoblast formation will allow us to use these proteins to develop tools to promote the regeneration of dental tissues.

In summary, we cloned MEPE cDNA by RT-PCR from human cDNA library, expressed MEPE proteins in *E. coli* cells, purified the recombinant protein, then we investigated the role of MEPE in dental pulp cells (DPSCs) ability of mineralization. We propose that this protein is involved in the increasing of HA at the mineralization of dentin and has a potential effect on dental rehabilitation.

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