Recombinant MEPE can increase hydroxyapatite *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 antibody, 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 a steady-state agarose gel system, and the results suggested that MEPE could induce hydroxyapatite (HA). We propose that this protein has a potential effect on dental rehabilitation.

Key words: MEPE, hydroxyapatite, steady-state agarose gel system.

INTRODUCTION

Recently, a new bone matrix protein cDNA was 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 MEPE (matrix extracellular phosphoglycoprotein), was isolated from a human oncogenic hypophosphatemic osteomalacia tumor (OHO) cDNA library (Rowe et al., 2000). MEPE, BSP (Bone sialoprotein), OPN (osteopontin), but sometimes known as SPP1 and Etal, DMP I (dentin matrix protein I), and DSPP (dentin sialophosphoprotein) 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 SIBLING family (Small Integrin-Binding Ligand N-linked Glycoprotein). And it is based not on current theories of their functions (which are poorly understood), but on the simple biochemical and genetic members. The human MEPE has 1989 bp cDNA clone features shared by all 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 (MacDouggall et al., 2002). 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 suggested that Dentonin affects primarily the initial cascade of events leading to pulp healing. In this study, we have cloned MEPE cDNA by RT-PCR, expressed MEPE in *E. coli* cells, purified the recombinant protein, and prepared anti-MEPE antibodies. Then western blot and immunohistochemistry were applied in the study. Finally, a steady-state agarose gels hydroxyapatite (HA) growth system was used to investigate the effects of MEPE to HA.

MATERIALS AND METHODS

MEPE cDNA clone isolation and amplifications

Human MEPE cDNA sequence from human brain cDNA library (BGI...
The cDNA clones were isolated in 50 µl reaction mixture using human MEPE primers (1 µl, 1 µmol/L, Forward: 5'-CGGAATTCAGAGCTATGTAAGGCT-3' with an EcoRI restriction site and 1µl, 1µmol/L, Reverse: 5'-CGGAATTCACCAGAGATTCTCAAAGATGCGAG -3' with an Hind III restriction site), high fidelity platinum Pfx DNA polymerase (0.5 µl, 1.5 U, Promega), 10 × PCR buffer (5 µl), MgCl₂ (1.5 µl, 50 mmol/L), dNTP Mix (1 µl, 10 mmol/L, Promega), human brain cDNA library (5 µl, denatured 99°C, 10min), 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 10 min for final extension and held at 4°C. Reaction products were analyzed by electrophoresis of 10 µl samples in 1% 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 DH5α. 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 analyses (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, and 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 Stratagene affinity cloning and protein purification kit.

SDS-page

Samples were mixed with the buffer (0.1% bromophenol blue, 20% glycerin, 2% β-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 with 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 (0.45l m, Bio-Rad, Chicago, IL USA) at room temperature using semi-dry blotting system (Millipore, Chicag, IL USA). Immunoblotting was performed using anti-MEPE antiserum 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 decaled in 10% EDTA for 2 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% H₂O₂ 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 diamino-benzidine substrate chromagen system according to the manufac-turer's instructions (Vector Laboratory).

ALP activity

The MEPE dose was varied between 0, 0.01, 0.1, 1, 10, 100 µg/ml to determine the ALP response on HDPC and HPDLC. At certain time points after treatment, cells seeded in 96 well plates were washed twice with 50 mM Tris-HCl (pH 7.3) and kept in 0.05% Triton X-100 lysis buffer overnight at 37°C. The ALP activity of the samples was determined by a colorimetric assay using an ALP reagent of p-nitrophenyl phosphate. The absorbance of p-nitrophenol formed by the hydrolysis of p-nitrophenylphosphate, catalyzed by ALP. The OD values were measured at 520 nm by a fusion plate reader. The enzymatic reaction was terminated with the addition of 3 ml of 0.1 N NaOH solution.

Effects of MEPE on HA formation in steady-state gels

Nucleation of HA was studied with a modified steady-state agarose gel previously described (Hunter and Goldberg, 1993). Agarose gels (1% agarose; volume approx. 1 ml) containing 0.15 M NaCl, 10 mM Tris/HCl, 0.01% NaN₃ and MEPE (50 µg) were poured into the central cavities of modified equilibrium dialysis cells. The control group did not add MEPE. Solutions containing 6.0 mM CaCl₂ or 6.0 mM sodium phosphate, pH 7.4, plus the above concentrations of NaCl, Tris/HCl and NaN₃ were pumped through chambers at either end of the agarose gels at a flow rate of 1 ml/h per gel. The entire apparatus was incubated at 37°C for various times (5 days). The gels were detected by X-ray diffraction analysis and then washed in concentrated nitric acid at 85°C, diluted with deionized water and analysed for phosphate by the phosphomolybdate method (Chen et al., 1956).

RESULTS

Isolation and recombinant of MEPE

In this study a human MEPE fragment of about 1.5 kb by
sequencing. A 1578 bp sequence was obtained. This was generated by RT-PCR from human brain cDNA library (Figure 1). Identity of the PCR product with the published human MEPE cDNA sequence was confirmed. The ATG codon at position 40 was selected as the translation initiation codon because it lies in the context of Kozak criteria (Kozak, 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 (Figure 2).

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 about 56 kDa (Figure 3A). 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 (Figure 3B).

Western blot analysis and immunohistochemical staining
The specificity of the anti-MEPE antibody was examined by 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 4A), demonstrating high specificity of the anti-MEPE antibody. Figures 4B, C, 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 4B).

ALP activity
The MEPE dose was varied between 0, 0.01, 0.1, 1, 10, 100 µg/ml to determine the ALP response, measured at day 10 of culture. Addition of MEPE to HDPC showed a significant increase in the activity of ALP. MEPE at a concentration of 100 µg/ml significantly increased ALP activity after 6 days of treatment. After 8 days of treatment, 100 µg/ml MEPE caused a 51% increase in ALP activity. So we think that the optimal dose had been achieved by 100 µg/ml. Having determined the optimal dose of MEPE, we then established the time course of its effect by measuring relative ALP activity. Over the first 4 days of treatment the effect of MEPE on HDPC cultured in mineralization media was undetectable. However, by the 6th day of treatment there began an increase in relative ALP activity that progressively rose over the next few days. By the 10th day of treatment, relative ALP activity was at its highest point indicating increasing HDPC mineralization over time in response to MEPE treatment. Meanwhile addition of MEPE to HPDLC did not show a significant increase in the activity of ALP, but ALP activity was increased by time manner (Tables 1, 2 and Figure 4).

Effects of MEPE on HA formation in steady-state gels
MEPE were incorporated at 5 - 25 µg/ml into steady-
### Table 1. HDPC ALP activity.

<table>
<thead>
<tr>
<th>Day</th>
<th>Co</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2</td>
<td>0.419 ± 0.026</td>
<td>0.426 ± 0.027</td>
<td>0.421 ± 0.002</td>
<td>0.439 ± 0.041</td>
<td>0.452 ± 0.014</td>
<td>0.466 ± 0.037</td>
</tr>
<tr>
<td>D4</td>
<td>0.427 ± 0.018</td>
<td>0.471 ± 0.016</td>
<td>0.537 ± 0.024</td>
<td>0.586 ± 0.004</td>
<td>0.627 ± 0.011</td>
<td>0.677 ± 0.021</td>
</tr>
<tr>
<td>D4</td>
<td>0.457 ± 0.007</td>
<td>0.51 ± 0.033</td>
<td>0.576 ± 0.025</td>
<td>0.639 ± 0.029</td>
<td>0.696 ± 0.009</td>
<td>0.768 ± 0.011</td>
</tr>
<tr>
<td>D8</td>
<td>0.473 ± 0.038</td>
<td>0.562 ± 0.026</td>
<td>0.618 ± 0.007</td>
<td>0.691 ± 0.020</td>
<td>0.751 ± 0.015</td>
<td>0.824 ± 0.022</td>
</tr>
<tr>
<td>D10</td>
<td>0.492 ± 0.011</td>
<td>0.583 ± 0.013</td>
<td>0.637 ± 0.024</td>
<td>0.703 ± 0.010</td>
<td>0.796 ± 0.041</td>
<td>0.904 ± 0.007</td>
</tr>
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</table>

**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 lanes 1 and 2. This is in approximate agreement with the expected molecular size. B: Immunohistochemical staining shows MEPE expression in dog grem tissue. 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.
Table 2. HPDLC ALP activity.

<table>
<thead>
<tr>
<th>Day</th>
<th>Co</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2</td>
<td>0.284 ± 0.021</td>
<td>0.293 ± 0.009</td>
<td>0.301 ± 0.010</td>
<td>0.313 ± 0.022</td>
<td>0.320 ± 0.011</td>
<td>0.334 ± 0.041</td>
</tr>
<tr>
<td>D4</td>
<td>0.296 ± 0.004</td>
<td>0.305 ± 0.026</td>
<td>0.317 ± 0.029</td>
<td>0.336 ± 0.003</td>
<td>0.343 ± 0.007</td>
<td>0.346 ± 0.036</td>
</tr>
<tr>
<td>D6</td>
<td>0.309 ± 0.019</td>
<td>0.358 ± 0.015</td>
<td>0.369 ± 0.007</td>
<td>0.387 ± 0.013</td>
<td>0.413 ± 0.028</td>
<td>0.421 ± 0.001</td>
</tr>
<tr>
<td>D8</td>
<td>0.336 ± 0.034</td>
<td>0.387 ± 0.043</td>
<td>0.409 ± 0.034</td>
<td>0.439 ± 0.019</td>
<td>0.473 ± 0.002</td>
<td>0.462 ± 0.021</td>
</tr>
<tr>
<td>D10</td>
<td>0.375 ± 0.012</td>
<td>0.486 ± 0.003</td>
<td>0.501 ± 0.028</td>
<td>0.553 ± 0.031</td>
<td>0.592 ± 0.035</td>
<td>0.582 ± 0.004</td>
</tr>
</tbody>
</table>

Table 3. Effects of MEPE on HA formation in steady-state gels.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>5 µg/ml</th>
<th>10 µg/ml</th>
<th>15 µg/ml</th>
<th>20 µg/ml</th>
<th>25 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.384 ± 0.042</td>
<td>0.413 ± 0.016</td>
<td>0.321 ± 0.057</td>
<td>0.403 ± 0.009</td>
<td>0.432 ± 0.023</td>
</tr>
<tr>
<td>MEPE</td>
<td>0.618 ± 0.046</td>
<td>0.862 ± 0.092*</td>
<td>1.067 ± 0.049*</td>
<td>1.259 ± 0.015*</td>
<td>1.287 ± 0.068*</td>
</tr>
</tbody>
</table>

MEPE were incorporated into agarose gels at 5, 10, 15, 20, 25 µg/ml and incubated for 5 days. Results shown are means ± S.D. Statistical significance was determined, compared with corresponding control values, using one-way analysis of variance and the Tukey multiple comparisons test (*P<0.001).

Figure 4. HDPC ALP activity was increased by time manner.

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 nucleic acid 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(SDSSSDDSSSDDSS), DMP1 (SSRRDDSSESSDSSSGSSSGS), osteopontin (DDSHQSDEHHSDSD), 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 a steady-state agarose gels HA growth system.

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. Peter-en 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 plays a signi-
Figure 5. MEPE were incorporated into agarose gels at various concentrations and incubated for 5 days prior to analysis for Ca and phosphate.

Figure 6. X-ray diffraction analysis of precipitates formed within steady-state agarose gels. A and B showed that HA were formed in the gels.
ficant 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 tissues especially in odontoblasts cell and predentin. These results indicated that during dentin development, MEPE might be involved in the initiation mineralization. There-fore, 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). ALP activity is considered a marker of HDPC and HPDLC mineralization because it generally increases during the mineralization stage. We observed a significant increase in ALP activity in cells treated with 100 µg/ml MEPE after 6 days of treatment on HDPC. Although this effect was not significant on days 2 and 4, probably due to the variability in ALP activity between samples within each group, a trend toward increased ALP activity in MEPE- treated cells was observed. The stimulating effect of MEPE on ALP activity indicates an increase in the mineralization of HDPC toward the mature dentine-forming phenol-type. Numerous studies with a variety of different experimental systems have attempted to determine the effects of mineralized tissue proteins on HA formation in vitro. The most exhaustively studied of these proteins is DPP. DPP has been shown to inhibit the formation of HA 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 formation of HA in a steady-state agarose gel system. Several workers have seeded growth of HA and on the amorphous calcium phosphate-HA transformation (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 HA 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 HA formation insolutions of low calcium phosphate supersaturation (Linde et al., 1989). No studies on the effect of MEPE on HA formation have been reported. In this study, the results reported here suggest that MEPE can induce HA. The potency of this effect is suggested by the observation at concentrations of MEPE as low as 5 µg/ml, with the maximal effect produced at 25 µg/ml. But proteins of mineralized tissues differ greatly in the ability to modulate HA formation in vitro. The observed effects of mineralized tissue proteins on nucleation and inhibition of HA formation suggest that proteins can modulate biological crystal formation in a variety of ways. The activities of MEPE suggest that is most likely to be involved in the initiation of mineralization in dentine respectively. In summary, we cloned MEPE cDNA by RT-PCR from human cDNA library, expressed MEPE proteins in E. coli cells, purified the recombinant protein, prepared anti-MEPE antibodies. We used an immunohistochemical approach and found that MEPE protein was expressed in dog dental germ tissues especially in odontoblasts, cell and predentin. Finally, MEPE has been shown to induce the formation of HA in a steady-state agarose gel systems, 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.

REFERENCES