The Effect of Matrix Extracellular Phosphoglycoprotein and Its Downstream Osteogenesis-related Gene Expression on the Proliferation and Differentiation of Human Dental Pulp Cells

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Abstract

Introduction: Matrix extracellular phosphoglycoprotein (MEPE), a new member of the small integrin binding ligand N-glycosylated (SIBLING) family, is believed to play multifunctional roles in regulation of cell signaling, mineral homeostasis, and mineralization. **Methods:** To study how MEPE affects the downstream genes involved in regulation of the proliferation and osteogenesis differentiation of dental pulp cells (DPCs), we explored the proliferation and osteogenesis differentiation capability of DPCs stimulated with recombinant MEPE or transfected with adenoviral-mediated human MEPE gene and used a systematic approach by osteogenesis real-time polymerase chain reaction arrays to profile osteogenesis-related gene expression after MEPE gene transfection. **Results:** Our results indicated higher proliferation capability in a time- and dose-dependent pattern by cholecystokinin octapeptide assay, and gene/protein expression of osteogenic markers bone sialoprotein, dentin sialophosphoprotein, osteocalcin, and collagen I were up-regulated dependent on time points showed by real-time polymerase chain reaction and Western blot. Moreover, a total of 3 genes, including enamelin, transforming growth factor-β2, and integrin α2, were significantly up-regulated. **Conclusions:** These results indicated that MEPE appeared to play an important positive role in proliferation and osteogenesis differentiation of DPCs through interaction with downstream signals. *(J Endod 2012;38:330–338)*

Key Words

Dental pulp cells, matrix extracellular phosphoglycoprotein (MEPE), osteogenesis, proliferation

Dentin-pulp complex is one of the main components of the tooth. The vitality of the dentin-pulp complex is fundamental to the functional life of the tooth, because it supports the defense reactions and the reparative events taking place in response to various noxious stimuli such as infection, exposure, trauma, and chemicals (1). Resident stem cell populations in dental pulp are thought to contribute to the regeneration and reparative process. Pulpal injury might send signals to stimulate the stem/progenitor cells migration, proliferation, and differentiation into odontoblasts. Dental pulp cells (DPCs) are heterogeneous population that contains progenitor cells at various differentiating stages in dental pulp, which might harbor great potential for reparation after pulp injury and tooth tissue engineering (2). However, the factors regulating the differentiation of DPCs into odontoblasts remain unclear. Studies have indicated that growth factors and extracellular matrix factors might be key groups of molecules for modulating a variety of cellular processes after dental injury (3–5).

Matrix extracellular phosphoglycoprotein (MEPE), also called osteoblast/osteocyte factor 45 (OF45), was first isolated from tumors associated with hypophosphatemic osteomalacia. As a new member of the small integrin binding ligand N-glycosylated (SIBLING) family of extracellular matrix proteins, similar to the other SIBLING proteins including osteopontin (OPN), dentin matrix protein 1 (DMP-1), bone sialoprotein (BSP), and dentin sialophosphoprotein (DSPP), MEPE is believed to be multifunctional, playing roles in cell signaling, mineral homeostasis, and mineralization (6). So far, its exact role in regulating mineralization and the underlying mechanisms is still controversial. Several studies have revealed the negative effect of MEPE on mineralization (7, 8), whereas others claimed the opposite (2, 9). MEPE is expressed in bone during the proliferation and early-maturation phases by fully differentiated osteoblasts, with maximal expression during mineralization (10). It is also expressed in immature odontoblasts and becomes down-regulated on odontoblastic differentiation (11). Because mice with MEPE ablated led to enhancement of bone formation, MEPE is believed to inhibit mineralization (12). However, when human DPCs were cultured in the osteogenic induction medium, MEPE mRNA expression increased in a time-dependent manner, and the result revealed a similar temporal expression profile with OCN and DSPP associated with the cellular differentiation (2). Also it is interesting to note that a mid-terminal fragment of MEPE (dentonin) has been found to enhance the proliferation of human bone marrow stromal cells and stimulate new bone formation (13) and dental pulp repair (14). Up to now, little is known about the exact functional role of intact MEPE in the DPCs and dental pulp regeneration and repair.

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In our previous studies, we have investigated the differential protein and gene expression profile of DPCs undergoing osteogenic differentiation by using 2D–difference gel electrophoresis (DIGE), mass spectroscopy–based proteomic approaches, and stem cell–related polymerase chain reaction (PCR) arrays, demonstrating that DPCs express a number of self-renewal and differentiation regulatory genes crosstalking with Notch, Wnt, transforming growth factor-β/bone morphogenetic protein, and cadherin signaling pathways (15, 16). In the present study to explore how MEPE affects the downstream genes involved in regulation of the proliferation and osteogenesis differentiation of DPCs, DPCs were stimulated with recombinant MEPE and further transfected with adenoviral-mediated human MEPE gene. A systematic approach by osteogenesis real-time PCR arrays was used to profile the osteogenesis-related gene expression after MEPE gene transfection to demonstrate the downstream signals of MEPE modulating osteogenesis differentiation of DPCs.

Materials and Methods

Human DPC Culture

Normal human premolars and impacted third molars were extracted from healthy young adults (12–28 years) undergoing orthodontic treatment in the Department of Oral and Maxillofacial Surgery, the Affiliate Stomatological Hospital of Sun Yat-sen University, after obtaining informed consent from each subject. The protocols were approved by the University Ethics Committee. DPCs were isolated and cultured as previously described (2). DPCs were cultured in Dulbecco modified Eagle medium (DMEM) (GIBCO-BRL Life Technologies, Breda, the Netherlands) supplemented with 20% fetal bovine serum (FBS) (GIBCO-BRL Life Technologies), 10 U/mL penicillin, and 10 mg/mL streptomycin (Sigma, St Louis, MO) and incubated at 37°C in 5% CO_2. The medium was changed every 3 days, and the cells at passage 3 were used for further study.

Effect of Recombinant Human MEPE on Cell Proliferation of DPCs

Cell proliferation was measured by cholecystokinin octapeptide (CCK-8) assay. Cells were grown in 96-well plates until they were 80% confluent. The cells were then starved in serum-free medium for 24 hours. Recombinant human MEPE (rhMEPE) (R&D System, Minneapolis, MN) was added to the medium at concentrations of 0, 2.5, 5, 25, 50, 250, and 500 ng/mL, respectively (n = 6 for each concentration). Cells were maintained in this medium for 24, 48, and 72 hours, respectively, and cell proliferation as measured according to the absorbance (490 nm) of reduced 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) was assessed by a quantitative assay (Cell Counting Kit-8; Dojindo, Kumamoto, Japan).

Effect of rhMEPE on Osteogenic Differentiation of DPCs

DPCs were induced in DMEM supplemented with 10% FBS, 10 mmol/L β-glycerolphosphate, 0.2 mmol/L ascorbate-2-phosphate, 100 nmol/L dexamethasone (osteogenic media; Sigma), combined with rhMEPE at the optimum concentration of 5 ng/mL as indicated by the above result. The cultures were maintained for 7, 14, and 21 days, respectively, and the medium was changed every 3 days. Cells cultured in normal medium without osteogenic induction (80% confluence) served as controls. The total RNA was extracted from DPCs by using the TRIzol Reagent (Qiagen, Valencia, CA). Assessment of the concentration and quality of the total RNA samples was carried out by spectrophotometry and gel electrophoresis. Complementary cDNAs were synthesized from 1 mg of total RNA by using a Omniscript RT kit (Qiagen) and RNase-free DNase Set (Qiagen) in a total volume of 20 mL according to the manufacturer's instructions. Five milliliters of reaction mixture was incubated with the PCR master mix containing double-stranded DNA dye SYBR Green I (Qiagen) in a total volume of 50 mL. Primers used for the quantitative PCR were as follows: BSP (forward: 5'-CAG AGG CAG AAA ACG GCA AC-3', reverse: 5'-TTC CGG TCT CTG TGG TGT CTT-3', 105 base pairs [bp]); DSPP (forward: 5'-GGG ATG TTG GCG ATG CA-3', reverse: 5'-CCA GCT ACT TGA GGT CCA TCT TC-3', 70 bp); OCN (forward: 5'-AGC AAA GGT GCA GCC TTT GT-3', reverse: 5' GCG CCT GGG TCT CTT CAC T-3', 63 bp); collagen type I (forward: 5'-CCT GCG TGT ACC CCA CTC A-3', reverse: 5'-ACC AGA CAT GCC TCT TGT CCT T-3', 84 bp); and β-actin (forward: 5'-GCA TGG GTC AGA AGG ATT CCT-3', reverse: 5'-TCG TCC CAG TTG GTG ACG AT-3', 106 bp). Conditions for reverse transcription PCR (RT-PCR) were 3 minutes at 93°C, followed by 40 cycles of 1 minute at 93°C, 1 minute at 55°C, and 1 minute at 72°C. Quantifications of mRNA were performed by using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the reported method (14). Each plate contained β-actin as housekeeping gene to normalize the PCR array data. All experiments were repeated 3 times. Raw data were acquired and processed to calculate the threshold cycle (Ct) value and relative gene expression values. Delta delta Ct method was performed to analyze mRNA expression levels.

Adenovirus Gene Transfection and Evaluation of MEPE Expression in DPCs

The CDS region of MEPE gene was amplified from a plasmid containing the full-length MEPE sequence and cloned into pDC316-mCMV-enhanced green fluorescent protein (EGFP) vector. After transformation into competent *Escherichia coli*, the candidate clones were identified by PCR and sequencing. The constructed recombinant shuttle plasmid pDC316-MEPE-EGFP and adenovirus helper plasmid pBHGlox_E1,3Cre were co-transfected into HEK293 cells in mediation of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and then propagated. Then a replication-deficient adenoviral vector type-5 Ad5-EGFP-MEPE, carrying the human MEPE gene and the reporter gene EGFP gene, was used in the current study. First, to acquire an optimum multiplicity of infection (MOI), DPCs were infected at different MOIs (50, 100, 250, 500, and 1000). Forty-eight hours after transfection, EGFP expression in DPCs was investigated under a fluorescence microscope, and the transfection efficiency was assessed.

After being infected at the achieved optimum MOI and cultured for 7, 14, and 21 days, the expression of MEPE in DPCs was examined by using real-time RT-PCR and Western blot. Real-time RT-PCR was applied as reported above. Primers used for the quantitative PCR were forward: 5'-CCC TGG AAG AGA AGG AAA CAG A-3', reverse: 5'-TGA AAC TCA ACC TTC CCT TGG T-3'. Western blot was performed as described previously (15). Briefly, the total protein was measured by a Bio-Rad Coomassie Blue protein assay (Bio-Rad Laboratories, Richmond, CA). Proteins were detected with mouse monoclonal antibodies against human MEPE (1:500 dilution; R&D) and human β-actin (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). All the experiments were repeated at least 3 times.

Effect of MEPE Overexpression on Cell Proliferation

Cells were grown in 96-well plates until they were 80% confluent. The cells were starved in serum-free medium for 24 hours and then transfected with Ad5-MEPE-EGFP and Ad5-EGFP (dilution in opti-DMEM) at the optimum MOI 250, respectively. The transfected and nontransfected cells were cultured for 1, 3, 7, and 10 days in DMEM supplemented with 10% FCS and 50 mg/mL gentamicin. Culture medium was changed at 3-day intervals. Cell proliferation was measured by CCK-8 assay as described above.