0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(8):4158–4161
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doi: 10.1210/jc.2003-032031

Serum Levels of Matrix Extracellular Phosphoglycoprotein (MEPE) in Normal Humans Correlate with Serum Phosphorus, Parathyroid Hormone and Bone Mineral Density.

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ABSTRACT. Matrix extracellular phosphoglycoprotein (MEPE), a member of the Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) family, is primarily expressed in normal bone and has been proposed as a phosphaturic factor because of high expression and secretion in oncogenic hypophosphatemic osteomalacia tumors. In order to begin to address the role of MEPE in normal human physiology, we developed a competitive ELISA to measure serum levels of MEPE. The ELISA was used to characterize the distribution pattern in a population consisting of 114 normal adult subjects. The mean value of MEPE was 476 ± 247 ng/ml and levels decreased significantly with increasing age. MEPE levels were also significantly correlated with serum phosphorus and parathyroid hormone (PTH). In addition, MEPE levels correlated significantly with measures of bone mineral density in the femoral neck and total hip in a subset of 60 elderly subjects. The results are consistent with MEPE being involved in phosphate and bone metabolism in a normal population.

Introduction

Matrix extracellular phosphoglycoprotein (MEPE), is a member of the SIBLING gene family (1, 2). Other family members include bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein-1 (DMP1), and dentin sialophosphoprotein. The family shares the RGD integrin-binding motif, several conserved phosphorylation and N-glycosylation sites, a common gene structure and chromosomal localization (4q21).

Normal MEPE expression has been described primarily in bone marrow, brain (3) and bone (4), while tumors which cause hypophosphatemic osteomalacia exhibit high expression and secretion (3). MEPE has consistently been linked with mineralization and bone formation associated with bone mineral (3-6). Whether MEPE plays a role as a positive or negative regulator of bone formation in humans remains unclear. The current study was undertaken to determine the distribution of MEPE in normal donors and to correlate the values with other biomarkers of bone metabolism as well as measures of bone mineral density (BMD).

Methods

Subjects. Sera from clinically defined normal patients were obtained under IRB approved protocols from a commercial serum bank (East Coast Biologicals, Inc., North Burwick, ME) as well as from the Johns Hopkins Bayview Medical Center General Clinical Research Center (JHBMC). The JHBMC normal group was obtained from an existing serum bank using samples from which all patient identifiers were removed.

Received 11/25/03. Accepted 3/22/04.

Cloning and expression of MEPE. The last exon of human MEPE constitutes 95% of the mature protein as defined by Rowe et al. (3). The last exon was amplified by PCR from human genomic DNA using a 5' oligonucleotide with a NdeI restriction site engineered in (AGTACCCATATGAAAGACAATA-TTGGTTTTCACCAT) and a 3' oligonucleotide with a BamHI site (CTGATGGGATCCCTAGTCACCAT-CGCTCTCAC). The ~1.5 kbp PCR product was gel purified, digested with NdeI plus BamHI, ligated into pET15b expression vector (Novagen, Madison, WI) digested with the same restriction enzymes. After transfection into BL-21 (DE3) E. coli cells, a high expression colony was selected and used to produce the MEPE protein by stimulation with IPTG. The MEPE protein in the apparent inclusion bodies was purified in 6 M urea on a HisBind resin column (Novagen) following the manufacturer's instructions. The eluted fraction was dialyzed exhaustively against 0.1 M ammonium acetate at 4°C and freeze-dried. Four ~200 μg aliquots of the highly purified MEPE were injected into a New Zealand white rabbit to make antiserum LF-155.

Serum sample preparation and competitive ELISA procedure. The SIBLINGs BSP, OPN and DMP1 are complexed with complement Factor H in human serum (7, 8). We have developed competitive ELISAs for measuring the BSP and OPN that requires disruption of the serum complex between the SIBLING and complement Factor H (9). For the current study, serum samples for use in ELISA analyses were processed in a chaotropic buffer exactly as described (9). The MEPE competitive ELISA developed utilized the same plates, buffers, and protein standard concentrations, secondary antibody concentrations, as well as substrate color reagents as

previously described (9). The only changes to the ELISA steps were that plates were coated with 10 ng/well recombinant MEPE and the primary antibody, LF-155, was employed at a 1:200,000 dilution.

Western blotting. Samples diluted in gel sample buffer were resolved by Tris/glycine SDS 12% polyacrylamide gels (Invitrogen, Inc., Carlsbad, CA) and transferred to nitrocellulose following standard conditions (10). Nitrocellulose membranes were rinsed with Tris-buffered saline-Tween (TBS-Tween, 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl containing 0.05% Tween 20). After a 1 h incubation in TBS-Tween + 5% non-fat powdered milk at room temperature, a 1:20,000 primary antibody (polyclonal antibody LF-155) was incubated overnight at 4 ^{O}C. The blot was washed in TBS-Tween four times for 5 min with TBS-Tween and then HRP-conjugated goat anti-rabbit IgG (1:2,000) in TBS-Tween + 5% milk was added and incubated for 2 h at room temperature. After washing, enhanced chemiluminescence reagents were employed for signal detection (Pierce Chemical Co., Chicago, IL) with x-ray film.

DEXA measurements. BMD was measured in the hip, spine and proximal femur using the Hologic QDR 1000 scanner (Hologic Corp., Waltham MA). The precision of this machine is 1.8% ± 0.05%. Mean values for total hip and spine were obtained, as were BMD values for neck and trochanter in the left proximal femur.

Serum and urine biochemical measures. Blood samples were drawn in the morning after an overnight fast. Serum bone biochemical measurements included bone-specific alkaline phosphatase (Hybritech, San Diego, CA), osteocalcin (Immunotopics, San Clemente, CA), procollagen type I carboxy-terminal propeptide (DiaSorin Stillwater, MN), intact PTH (Nichols Institute, San Juan Capistrano, CA), and 25 hydroxy vitamin D (DiaSorin). The excretion of deoxypyridinoline crosslinks (Quidel Corp. San Diego, CA) and cross-linked amino-terminal telopeptides (OSTEX International, Seattle, WA) were assayed in 2-hour, second-void morning urine specimens. The values for cross-links were normalized to urinary creatinine assayed using the Jaffe Rate method and a Beckman Creatinine Analyzer 2 (11). Serum inorganic phosphorus was measured using standard clinical methods (12). The performance characteristics of the immmunoassays as carried out in our laboratory are given in Table I.

Results

Previous work has demonstrated that the SIBLINGs BSP, DMP1 and OPN were bound to complement Factor H in serum (7, 8). Disruption of the serum complex required heating in a chaotropic buffer containing reducing agent, followed by a column step to clean up the sample (9). The same disruption procedure was used on serum samples from elderly and young adult donors and MEPE was detected by western blot. Immunoreactive bands shifted in migration with reduction and younger donors appeared to have more MEPE present (Fig. 1a and b). The amount of MEPE present in sera from 114 different normal subjects was analyzed. A reproducible standard curve profile combining 34 different analyses performed over the past two years was obtained (Fig. 1c). MEPE values quantified by ELISA paralleled semi-quantitative results obtained from western blots (Fig/ 1c, inset).

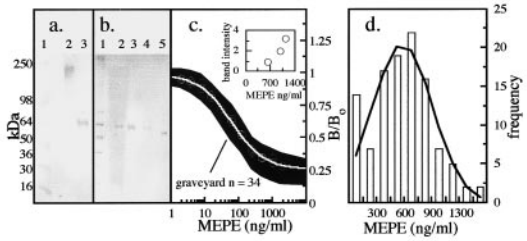

Figure 1. MEPE is present in human serum. (a) Unreduced (lane 2) and reduced (lane 3) serum samples from normal donors were analyzed for the presence of MEPE by western blot. Molecular weight standards were run in lane 1. (b) Samples derived from different age donors analyzed by western blot. Lane 1, standards; lanes 2, 3 & 4 contained 60, 24 and 78-year-old normal donor serum, lane 5, recombinant MEPE (lacking glycosylation). (c) Competitive ELISA profile; inset, representative correlation of western blot band intensity with ELISA results. (d) The distribution of MEPE (bars) in 114 normal subjects. Solid line represents the normal Gaussian distribution.

Addition of recombinant MEPE to serum samples prior to reduction, column chromatography and competitive ELISA yielded an average recovery of 88 % based on three different trials. The inter-assay coefficient of variance for repetitive measurements on the same serum sample was 19.4% (n = 6), while the intra-assay coefficient of variance was 12.6% (n = 12). The major source of this variance was tracked to the column chromatography step. Repeated measures of post-reduction and column samples gave rise to a coefficient of variance of 7.9%. The measure of MEPE levels in normal subject-derived sera revealed a distribution with a slight hook at the low end (Fig. 1d).

When MEPE levels were plotted versus the age of the subject, the reason for the low end hook to the distribution of normal values became apparent. MEPE exhibited a significant age-related decrease in level, (Fig. 2a). The population analyzed possessed a sufficient number of subjects > 60 years of age, where MEPE levels are 1/2 to 1/3 those of younger adults, to account for the increased distribution at low MEPE levels. Serum measures of markers of bone metabolism were also performed on normal subjects. Comparing MEPE levels with serum values of bone-specific alkaline phosphatase, procollagen type I carboxy-terminal propeptide, 25-hydroxy vitamin D, osteocalcin, and urine levels of collagen cross-link markers revealed no significant correlation (data not shown).

Table I. Immunoassay Performance and Study Population Characteristics.

Analyte		mean ± s.d.	units	range	%CV intra-assay	%CV inter-assay
bone-specific alkaline phosphatase		11.2 ± 4.2	ng/ml	5.0 – 28	5.49	5.83
deoxypyridinoline crosslinks		5.1 ± 2.0	nM/mM Cr	1.7 – 13	6.00	4.16
N-terminal telopeptides		31.3 ±13.3	BCE/mM Cr	5.2 – 64.2	8.25	4.00
osteocalcin		5.2 ± 1.9	ng/ml	2.1 – 10.2	4.55	6.10
procollagen type I C-terminal propeptide		133.9 ±45	ng/ml	10.7 – 289	2.24	4.38
intact parathyroid hormone		33.7 ± 14.5	pg/ml	3.2 – 94.2	2.40	5.95
25-hydroxy vitamin D		34.5 ± 9.3	ng/ml	14.6 – 62.6	5.19	7.90
MEPE		476.0 ± 247	ng/ml	19.0 – 1269	12.60	19.40
study population	male (n = 64)	60 ± 20	years	21 - 87		
	female (n = 54)	55 ± 12	years	35 - 62		
	BMD group (n = 60)	65 ± 11	years	50 - 82		

Because MEPE has been proposed to play a role in phosphate metabolism, we next investigated serum levels of PTH and inorganic phosphorus. Using a third generation commercial intact PTH assay, the PTH levels were found to be significantly negatively correlated with serum MEPE values (Fig. 2b). The serum levels of intact PTH showed no correlation with donor age (data not shown), suggesting that the association of MEPE and PTH was age-independent. The levels of serum phosphate in the same donors was significantly positively correlated ($r^2 = 0.35$, $p \leq 0.0001$) with serum MEPE values (Figure 2b, inset).

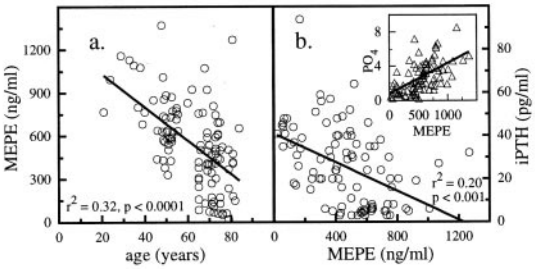

Figure 2. MEPE levels in serum correlate with age (a) and parathyroid hormone levels (b). Serum inorganic phosphorus levels were also determined (inset).

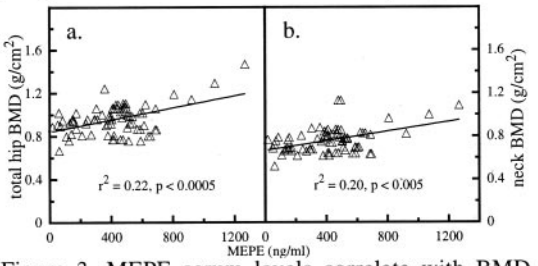

Figure 3. MEPE serum levels correlate with BMD values for total hip (a) and femur neck (b).

In addition to serum and urine markers of bone metabolism, BMD measurements were obtained on a subset of normal subjects (n = 60). The BMD values (g/cm^2) determined were analyzed for correlation with MEPE levels in the corresponding subject's serum (Fig. 3). MEPE levels were significantly positively correlated with bone mineral density values for total hip and femur neck. MEPE levels were also correlated with femur trochanter BMD ($r^2 = 0.13$, $p \leq 0.01$), while the correlation with total spine BMD did not reach statistical significance (data not shown). The correlation of serum MEPE levels with BMD was still significant when adjusted for subject age using multiple regression analysis and StatView software (SAS Institute, Inc.)

Discussion

MEPE and its rodent homologue, OF45, have been implicated in bone and mineral metabolism (3, 4, 13). The increase in bone density found in the OF45 knockout mouse in the presence of normal serum phosphorus and calcium without evidence of a mineralization defect, suggests that it may have a direct effect on bone formation (6). In that report, the MEPE knockout animal had, what is for a gene knockout model, a relatively subtle increase in the amount of bone. While histomorphometric analysis was performed, there were no data on the parameters of mineralization (osteoid thickness, etc.), only formation and resorption. Elevated levels of MEPE mRNA expression by tumors from patients with hypophosphatemia and osteomalacia suggested that it may be involved in mineral homeostasis. The control of systemic phosphate homeostasis is incompletely understood. Key modulators include PTH, calcium, phosphorus, vitamin D, as well as novel phosphatonin(s), and the bone and kidney organs. Candidate phosphaturic factors include MEPE; PHEX, a putative endopeptidase believed to process factors regulating bone mineralization and renal phosphate reabsorption (5); FGF23, a phosphaturic factor in fibrous dysplasia (14), tumor-induced osteomalacia and autosomal-dominant hypophosphatemic rickets (15, 16) and secreted frizzled-related protein 4, an antagonist of renal Wnt-signaling (17). These phosphate regulators remain to be fully characterized both individually, and in their interactions which will lead to the description of a new hormonal pathway.

Demonstration of significant levels of MEPE in the serum of normal humans, as well as a clear age-related decrease suggest that MEPE may be an interesting marker of normal human bone and mineral metabolism. While the positive correlation between MEPE and phosphorus might suggest an anti-phosphaturic effect, it may represent a secondary response to higher serum phosphorus levels. This idea is supported by the significant correlation of serum MEPE levels with the important constituents of mineral metabolism serum, PTH and phosphorus. The relationships between serum MEPE and PTH, MEPE and phosphorus, and phosphorus and PTH are all internally consistent, and the relationship between phosphorus and PTH is consistent with established

physiology. The correlation of MEPE levels with BMD suggests that it may be involved in mineralization in the human. The finding that MEPE is low in aged patients, when BMD is lower, and that MEPE levels are higher when BMD is high is corroborative, and suggests that these findings in humans are of physiologic significance. Two recent studies have provided contrasting data on the biological activity of MEPE. Recombinant MEPE promoted renal phosphate excretion in mice and inhibited BMP2-mediated mineralization in a mouse osteoblasts cell line (18). The inhibitory action was mapped to the carboxy terminal region of the molecule. In a second study, a peptide fragment corresponding to the RGD-containing mid region stimulated new bone formation in neonatal mouse calvarial organ culture and increased osteoblast proliferation and alkaline phosphatase activity (19). Our current study demonstrates the association of serum MEPE levels with serum phosphate, PTH and bone mineral density but does not address causality.

Acknowledgements

This research was supported by CA 87311 (N.S.F.), DAMD17-02-0684 (N.S.F.) and the Johns Hopkins Bayview Medical Center General Clinical Research Center, NIH/NCRR grant M01RR02719.

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