Expression of rhMEPE in E. coli

To construct an expression plasmid for the mature form of human MEPE [Ala17-Asq525](4) in *E. coli*, the PCR reaction with *Pyrovest* DNA polymerase was carried out using pCR-MEPE-9 as a template. The forward and reverse primers were 5'-CATATGGCACCAACATTTCAACCACAGA-3' containing *Nde* I site (underlined) and initiation codon ATG and 5'-CTCTCGTCGACATCAACTCACA-3' of 3'-non-coding region of MEPE cDNA, respectively. The PCR fragment was inserted in pCR-Blunt vector (Invitrogen) to obtain pCR-mMEPE. The pCR-mMEPE plasmid was then digested with *BamH* I and *Nde* I as the *BamH* I site was in the pCR vector region, and th excised fragment was inserted into the *Nde* I-*Bam*H I cloning site of the pTCII vector (12) to produced pTCII-mMEPE-2. The nucleotide sequence of the cDNA for the mature MEPE thus obtained was confirmed in a 377 DNA sequencer (Applied Biosystems).

E. coli MM294 (DE3) was transformed with the pTCII-mMEPE-2 plasmid and cultured with M9 medium. The production of rhMEPE in *E. coli* cells was induced with IPTG under the control of T7 promoter.

Purification of rhMEPE

rhMEPE produced in *E. coli* cells did not form inclusion bodies and was easily extracted by sonication of *E. coli* cells suspended in 50 mM MES buffer, pH 6.0. The recombinat protein was purified from the cell-free extract by cation exchange chromatography on SP-Toyopearl 550C (Tosoh, Tokyo, Japan), cation exchange HPLC with a CM-5PW column (Tosoh), and gel filtration HPLC with a Superdex 200 column (Amersham Biosciences Corp., Piscataway, NJ, USA). The purification was monitored by SDS-PAGE and Coomassie Blue staining (Fig. 1). Using a model 492 Procise protein sequenator (Applied Biosystems), the purified products were subjected to N-terminal sequence analysis after protein transfer to Immobilon P membranes (Millipore). The products were also analyzed by mass spectrometry with a MALDI-TOF mass spectrometer (Voyager DE, Applied Biosystems) to determine molecular weights.

Preparation of polyclonal anti-MEPE antibody and immunoblotting

A rabbit was immunized with rhMEPE protein (1mg) in Freund's complete adjuvant (Wako,Osaka,Japan), which was injected at multiple subcutaneous sites in the back and intramuscularly into both thighs. After 2,4,6, and 8 weeks, the rabbit was given the half dose (0.5mg) of rhMEPE in Freund's incomplete adjuvant (Wako). One week after the final booster injection, the rabbit was sacrificed to obtain 70 ml of anti-serum. The anti-serum had a titer of around 10^6-10^7 when assayed by EIA using HRP-conjugated anti-rabbit IgG (Wako).

The anti-serum (6 ml) was diluted two-fold with MAPSII binding buffer (Bio-Rad Laboratories Inc., Tokyo, Japan), and applied to a column of Protein A-Sepharose FF (1.6 x 5.0 cm, 10ml, Amersham Biosciences Corp.). An IgG fraction was eluted from the column with MAPSII elution buffer (Bio-Rad Laboratories Inc.) followed by neutralization. After dialysis against PBS, the IgG fraction was chromatographed on a column of MEPE-coupled NHS-Hitrap(1 ml, containing 3mg of rhMEPE coupled). The specific antibody fraction was eluted with 0.5 M NaCl-0.1M glycine-HCL (pH 2.7). After neutralization and dialysis against PBS, the affinity-purified anti-MEPE IgG fraction was stored at 4° until use. Starting from 6 ml of anti-serum, 16 mg of rabbit plyclonal anti-MEPE antibody was obtained.

The *E. coli* cells expressing rhMEPE described above were cooked with the SDS-PAGE sample buffer to prepare a cell lysate, which was subjected to Western blotting using the anti-MEPE IgG and HRP-conjugated goat anti-rabbit IgG(Wako) as the first and second antibody, respectively.

Mammalian expression of human MEPE

To amplify the full length of an open reading frame (ORF) in human MEPE cDNA, PCR with *pyrovest* DNA polymerase was carried out using pCR-MEPE-9 as a template. The forward and reverse primers were 5'-CTCAAAGATGCGAGTTTTCTGTGTGGGA-3' containing ATG(underlined) that corresponds to the original initiation codon in the ORF and 5'-CTACTTATCGTCGTCATCCTTGTAATCGTCACCATCGCTCTCACTTGA-3' containing the FLAG sequence inserted in front of the translation stop codon. The amplified gene was subcloned into the CMV mammalian expression vector pTARGET (Promega, Madison, WI) to obtain pT-MEPE-11 and the sequence of the gene thus obtained was confirmed using a 377 DNA sequencer (Applied Biosystems). Trasfection of pT-MEPE-11 into CHO cells was carried out with LipofectAMINE (Invetrogen) for 3 hours. The culture medium was exchanged to a fresh one, collected after the culture for 24 hours, and subjected to immunoblotting with the anti-MEPE antibody.

Clinical profile of the patients

Samples of normal bone tissue were obtained from four patients during surgery after they gave informed consent. These patients included an 18-yr-old man with fracture of the tibia (NC-1), an 80-yr-old woman with fracture of the femoral neck (NC-2), a 69-yr-old woman with osteoarthritis of the knee (NC-3), and 2-yr-old boy with below-knee amputation for fibrosarcoma of the tibia (NC-4)(we used the intact fibula for the present study).

Bone samples exhibiting osteomalacia were obtained from four patients who were diagnosed by laboratory tests and iliac bone biopsy. Two patients had OHO(OM-1 and OM-4), one had Fanconi's syndrome (OM-2), and one had vitamin D-deficient rickets (OM-3). The characteristics of these patients are summarized in Tale 1.

Tissue Preparation

Paraffin sections: Decalciffed paraffin sections were prepared for immunohistochemistry to detec MEPE expression. Tissue samples were fixed in 4% paraformaldehyde (pH 7.4) at 4℃ for 24 h, decalcified in 20% EDTA (pH 7.4), dehydrated through an ethanol series and finally embedded in paraffin. Then the specimens were cut into serial sections(5 μm thick)on a microtome, mounted on slides, and prepared for immunohistochemistry. One of the sections was stained with hematoxylin and eosin to assess the histological feature of each bone sample.

Methylmethacrylate (MMA) sections: Sections were also prepared from non-decalcified tissue to distinguish between calcified and non-calcified areas and to define the immunolocalization of MEPE. Iliac bone samples from the four osteomalacia and four osteoporosis patients were fixed in 70% ethanol, prestained with Villanueva bone stain for 7 days, dehydrated through an ethanol and acetone series, and then embedded in MMA, as described previously (13). Dry sections (5 μm thick) were cut to distinguish non-calcified osteoid as Villanueva-positive areas using a Jung Supercut 2065 Microtome(Leica Microsystems, Heidelberg, Germany)equipped with a tungsten carbide knife. For immunohisochemistry, serial wet sections (5 μm thick) were cut in the same way while applying 30% ethanol to the block and knife. These sections were carefully stretched using 70% ethanol, mounted on gelatin-coated slides using a mixture of carbolic acid crystals and glycerol, flattened with a rubber roller, pressed with a slide press, and dried on a hot plate at 40℃.

Immunohisochemical staining

Paraffin-embedded tissue sections were deparaffinized, while MMA-embedded sections were deacrylated in acetone for 8 min twice an decalcified in 20% EDTA(pH 7.4) for 1 h. After rinsing with water for 10 min, the sections were incubated in 0.3% H_2O_2 in 90% methanol for 30 min at room temperature to block endogenous peroxidase activity, and then in 10% normal goat serum to minimize nonspecific background staining. Then the rabbit polyclonal antibody directed against human MEPE was applied to each section and incubated overnight at 4℃. An isotype-matched antibody was used for control staining. Next, detection was performed using the streptavidin biotin-peroxidase complex technique (Histofine SAB-PO Kit; Nichirei Co., Tokyo, Japan). Finally the sections were developed in 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) and counterstained with hematoxylin.

Analysis of the MEPE positivity of osteocytes

MEPE positivity of osteocytes was analyzed in mineralized bone tissue and in non-mineralized osteoid from all bone samles. Sections with immunostaining for MEPE, as well as Villanueva-satined MMA-embedded sections, were examined under a light microscope(ECLIPSE E1000,Nikon Corporation, Tokyo,Japan). Five randomly chosen visual fields at 200 x magnification were examined, and the mean value was calculated. First, the number of osteocytes was counted in the mineralized or non-mineralized areas of the Villanueva-stained sections. Then the number of MEPE-positive cells was counted in the immunostained serial section for the areas that corresponded to those characterized as mineralized or non-mineralized by Villanueva sataining. Thereafter, MEPE positivity was calculated as the ratio of MEPE-positive osteocytes to the total number of osteocytes in the mineralized area and the non-mineralized osteoid. Results are presented as the mean ± SD. Statistical analysis was performed with the Mann-Whitney U test and $p<0.05$ was considered significant.

RESULTS

Cloning and expression of human MEPE

A cDNA library was prepared with a nasal tumor tissue causing OHO. By using the cDNA library as a template, a 1.7 kb fragment containing an open reading frame of human MEPE was amplified by PCR using a forward primer derived from the cloning vector and a reverse primer located in 3' untraslated region of MEPE cDNA. To express the mature form of human MEPE, a cDNA fragment encoding the polypeptide [Ala17-Asp525] with an additional methionine residue attached at the N-terminus for an initiation condon was amplified by PCR and subcloned in an expression vector to obtain pTCII-mMEPE-2. *E. coli* MM294 (DE3) was transformed with the expression plasmid and expression of MEPE was induced with IPTG to yield 67 kDa of a soluble protein. The expressed MEPE was then extracted from the *E. coli* cells by sonication and purified by 2 steps of cation-exchange chromatography on SP TOYOPEARL 550C and CM-5PW columns followed by gel-permeation chromatography on a Superdex 200 column. Starting from 50g of *E. cloi* cells, 30mg of the rhMEPE protein was obtained.

As monitored by SDS-PAGE (Fig. 1), the purification of rhMEPE resulted in a product split into 2 closely associated protein bands. Each band was separated and subjected to N-terminal sequence analysis and mass spectrometry using a MALDI-TOF mass spectrometer. N-terminal sequences of 2 bands were found to be identical to that deduced from the cDNA sequence, i.e., Met-Ala-Pro-Thr-Phe-Gln-Pro. The molecular weight of the upper band was determined to be 56,730 Da, which was well consistent with a theoretical molecular weight of rhMEPE, 56,723.08 Da predicted from the cDNA sequence. The molecular weight of the lower band was 54,150 Da, which suggests deletion of C-terminus 24 residues (△2,539 Da)probably during the extraction by sonication and /or the purification.

Western blot analysis of rhMEPE using polyclonal antibody

The affinity-purified anti-MEPE IgG was prepared by using the rhMEPE protein as described in Materials and Methods. Specificity of the anti-MEPE antibody was examined first by Western blotting of a crude cell lysate of the *E. coli* expressing

rhMEPE (Fig. 2). rhMEPE immunoreactivity was visualized as a single band at 67 kDa (lane 2) to demonstrate high specificity of the anti-MEPE antibody. Further, the anti-MEPE antibody was tested with immunoblotting of culture medium from CHO cells transiently transfected with an MEPE expression plasmid (Fig. 2). A secreted protein of 70 kDa was selectively detected (lane 3), confirming the specificity of the antibody. A faint and broad signal was also observed at the position of 100-150 kDa, indicating the secretion of the heavily glycosylated forms of MEPE from the CHO transfectants.

Uniform MEPE expression in normal mineralized bone

MEPE expression was examined in normal bone tissue. Figure 3 shows a sample of adult bone (NC-1, tibial fracture), and Figure 4 shows a sample from a child (NC-4 intact fibula). The other two samples (NC- and NC-3) showed similar features to those of NC-1. MEPE was strongly expressed by osteocytes, including both cortical bone and trabecular bone (Figs. 3-B and F), while it was not expressed by osteoblasts (Fig. 4-C). The pericellular bone matrix and the dendritic processes of osteocytes and preosteocytes, which were partly embedded in the bone matrix, were also strongly stained (Figs. 3-D, H, and J), but no staining of the bone-lining cells was observed (Fig. 3-J).

Focal MEPE expression in bone tissue from osteomalacia patients.

Bone tissue from osteomalacia patients was also examined. The clinical characteristics of these patients are shown in Table 1. Figure 5 shows the features of bone tissue from one patient(OM-1,OHO), and the other three patients revealed similar findings. MEPE expression was mainly observed in the osteocytes in cortical bone, although focal expression was seen in trabecular bone (Fig. 5-B); expression was more abundant in the central area than at the boundary zone, which could be regarded as osteoid (Fig. 5-E).

MEPE expression is localized to mineralized bone in patients with osteomalacia

In order to assess the expression of MEPE in osteoid, we performed Villanueva staining of bone tissue specimens from osteomalacia patients and MEPE

immunostaining using serial sections. Figure 6 shows the results obtained in one patient (OM-1, OHO) and the other three patients revealed similar findings. Villanueva staining revealed marked osteoid formation at the boundary zone of trabecular bone (Figs.6-A and B). MEPE expression was observed in trabecular bone, except in the Villanueva-stained non-mineralized osteoid area (Fig. 6-C). From these findings, MEPE seemed to be almost entirely expressed in mineralized bone tissue.

MEPE expression is localized to mineralized bone in patients with osteoporosis

In order to confirm the different patterns of MEPE localization between bone tissue with or without disturbance of mineralization, iliac bone specimens from four osteoporosis patients were examined by the same method as the osteomalacia tissue (Fig. 7). It was found that osteocytes within the osteoid area showed little MEPE positivity, as was the case in osteomalacia (Fig. 7-C). All four patients showed similar findings.

MEPE expression is largely limited to osteocytes in mineralized bone tissue

MEPE positivity was calculated as the ratio of the number of positive osteocytes to the total number of osteocytes in each subject. In bone samples from patients with osteomalacia or osteoporosis, it was calculated separately for the mineralized area and the non-mineralized osteioid. Normal bone tissue showed approximately 94.2% MEPE positivity (Table 2), while the mineralized area of bone from osteomalacia patients showed 87.5% positivity (Table 3) and the mineralized area of osteoporotic bone showed 95.3% positivity (Table 4). In contrast, the non-mineralized osteoid in bone from osteomalacia patients only showed 7.8% positivity (Table 3), while that from osteoporosis patients showed 4.9% positivity (Table 4). These results confirmed that MEPE is almost exclusively expressed by the osteocytes in mineralized bone *($^*p < 0.05$),* and that there is no significant difference between bone samples from patients with osteomalacia and osteoporosis.

DISCUSSION

In this study, we prerformed cDNA cloning of human MEPE from the cDNA library of a human nasal tumor causing OHO. Then we expressed the cDNA in *E. coli* and purified the expression product. In SDS-PAGE the product split into 2 closely associated protein bands (Fig. 1). However, the N-terminal sequences of both bands were conseistent with that deduced from the cDNA sequence of rhMEPE, showing that both proteins are indeed derived from rhMEPE. The result of the mass spectrometry, *i.e.,* the good agreement of the molecular weight with the theoretical value, proves that the polypeptide of a highter molecular weight is an intact rhMEPE. The product with lower molecular weight appears to be rhMEPE with C-terminus deletion during the extraction by the sonication and/or purification. By using this product we developed a rabbit polyclonal antibody against MEPE and prepared affinity-purified anti-MEPE antibody. Specific detection of crude rhMEPE derived from *E. coli* and CHO cells in the Western blotting with the anti-MEPE antibody (Fig. 2) demonstrates its excellent specificity for MEPE. The rhMEPE with C-terminus deletion was not detected in the immunoblotting of a cell lysate directly prepared from the *E. coli* by the rapid heat denaturation in the presence of SDS and DTT. The difference in the molecular weight between the *E. coli* and the CHO transformants implies the glycosylation of MEPE in CHO cells.

Using the anti-MEPE antibody, thus obtained, we investigated MEPE expression in normal human bone tissue by immunohistochemical analysis to assess the distribution of MEPE, and recognized the predominant expression of MEPE by bone-embedded osteocytes. In normal adult bone tissue, MEPE protein was strongly expressed by bone-embedded osteocytes and pre-osteocytes, including their processes, as well as in the pericellular bone matrix of these osteocytes (Figs. 3A-3H), while osteoblasts (Fig.4) and bone-lining cells (Figs. 3I-3K) did not express MEPE. This is the first report on MEPE expression in human bone at the protein level and our findings were compatible with the results already obtained in mice (9) and rats (8). Positive immnostaining of osteocytes, but not osteoblasts, suggested that MEPE protein is produced by mature osteoblasts during the process of embedding in new bone matrix and mineralization throughout osteocyte development, as already

mentioned by Gowen et al.

Argiro et al. Performed an in vitro study on mouse bone cells and found that MEPE mRNA was expressed by fully differentiated osteoblasts and that its expression increased markedly during osteoblast-mediated mineralization of matrix (7). A close correlation between MEPE expression and mineralization after the addition of glycerophosphate to osteoblast culture medium was also reported by Peterson et al, so we focused on non-mineralized osteoid in human bone and examined the details of MEPE expression in the non-mineralized bone of osteomalacia and osteoporosis patients. Our findings show that MEPE is not expressed by osteocytes in non-mineralized osteoid (Fig.6, 7), but is abundantly expressed by osteocytes in mineralized bone matrix (Fig. 3, 6, 7). Our results and previous in vitro data suggest that MEPE is highly expressed in the mineralization phase as osteoblasts undergo maturation into osteocytes. However, it is not clear whether MEPE expression precedes mineralization or is preceded by mineralization of the bone matrix.

The non-mineralized osteoid area is too small in normal bone to detect osteocytes in the non-mineralized bone matrix by standard hisological examination. This may be the reason why in previous studies did not immunohistochemically detect osteocytes without MEPE expression in normal rodent bone. Preparing bone samples from osteomalacia patients and using a combination method of hitomorphometry and immunohistochemistry as reported by Derkx et al. made it possible to examine the osteoytes in non-mineralized bone matrix.

Recently, Gowen et al. clearly showed that MEPE plays an inhibitory role in bone formation using knockout mice. They showed that an increase of osteoblast numbers and activity in knockout mice resulted in an increase of bone mass. Van Bezooijen et al. also reported that some proteins produced by osteocytes may have an inhibitory effect on osteoblastic activity (14). They showed that sclerostin, the SOST gene product, produced by osteocytes could inhibit the effect of BMP on mature osteoblasts and calcium deposition in vitro. These new data suggest that proteins produced by osteocyte may control bone remodeling and that MEPE is one of these osteocyte-derived proteins.

On the other hand, Argiro showed that MEPE mRNA expression during osteoblast-mediated matrix mineralization was increased in Hyp osteoblasts rather than

directly associated with the development of mineralization disorders. Further investigations are needed to assess the correlation between MEPE and osteomalacia in relation to its effect on bone metabolism.

Osteocytes are easily defined by their location and typical stellate morphology, and have relatively few organelles that are necessary for matrix production and secretion (25-26). During the last decade, several non-collagenous matrix proteins have been found in and around osteoblasts and osteocytes, including OPN(27-31), osteocalcin (32-34), BSP (35-37), biglycan (38-40), osteonectin (41), and DMP1 (42-44). These proteins are thought to play various roles both in promoting bone mineralization (43) and in the attachment of osteocytes to the bone matrix (46), and knockout gene studies have supported these theories (47-51). Some of these proteins belong to the SIBLING family (10), which share many unique motifs and structural features (such as the RGD motif and ASARM motif encoded on chromosome 4q) and are considered to have similar functions (4). Since these proteins are expressed by aboth osteocytes and osteoblasts, few osteocyte-specific markers have been established apart from DMP1 and several monoclonal antibodies directed against avian osteocytes (MAb OB7.3 (52), MAb OB37.11(53), and MAb SB5 (54)). Recently, MAb OB 7.3 was proved to target Phex protein (55). MEPE was already proved to be expressed by rodent osteocytes, but not osteoblasts in immunohistochemistry and we demonstrated its predominat expression in osteocytes from mineralized bone matrix. These findings make it possible that MEPE is a novel specific marker for osteocytes in mineralized bone.

In summary, we performed cDNA cloning of human MEPE from the cDNA library of a human nasal tumor tissue causing OHO. Then we obtained rhMEPE and developed a rabbit polyclonal anti-MEPE antibody. Using this antibody, we demonstrated that MEPE protein is expressed by osteocytes in human bone specimens. We also investigated MEPE expression in bone tissue from patients with osteomalacia and osteoporosis, and found that its expression by osteocytes is localized to mineralized bone. Further investigations are necessary to elucidate the functions of this extracellular bone matrix protein.

wild-type osteoblasts(7). Hyp mice have deletion of the Phex (phosphate-regulating gene with homologies to endopeptidases on the X choromosome) gene, and are used as an animal model of XLH (X-linked hypophosphatemic rickets) (15). Recently XLH, ADHR (autosomal dominant hypophosphatemic rickets), and OHO have been viewed as due to problems of Phex and FGF-2 (16-17). FGF-23 was reported to be one of the tumor-derived factors that can cause hypophosphatemic osteomalacia. It was also suggested that FGF23 is cleaved into an inactive form by the Phex gene product. In XLH, FGF-23 is no cleaved by the mutant Phex gene product, resulting in an increase of FGF23 activity and hypophosphatemic osteomalacia. In ADHR, mutant FGF-23 is resistant to proteolytic processing and, resulting in an increase of FGF23 activity and hypophosphatemic osteomalacia (18-19). Recent reports have referred to an association between MEPE and Phex, showing that the Phex gene product inhibits proteolytic cleavage of MEPE by cathepsin B (29). Given that MEPE knockout mice show an increased trabecular bone mass, the possibility has been raised that MEPE degradation products are responsible for inhibiting mineralization in the setting of abnormal Phex.

MEPE, FGF-23, and frizzled-related protein-4 (FRP-4) have been detected in OHO tumors and are regarded as candidate phosphatonins, which are thought to cause hypophoshatemic osteomalacia (5). FGF-23 was demonstrated to act as a phosphatonin based on induction of hypophosphatemia (21); and after resection of the tumor in an OHO patient, the elevated serum level of FGF-23 rapidly fell to normal together with resolution of symptoms and normalization of other parameter, including serum phosphate, urine phosphate, and serum calcitriol(22). FRP-4 was also reported to inhibit renal phosphate reabsorption (23-24). In the case of MEPE, Shimada et al. reported that mice with CHO cells secreting MEPE did not show hypophosphatemia (21), but Rowe et al. reported that MEPE has a role as a phosphaturic factor (6). MEPE may possibly be associated with osteomalacia as a phosphatonin by preventing renal phosphate reabsorption.

It remains unclear whether MEPE has a direct role in osteomalacia at the level of bone metabolism. Our study could not detect a difference in the distribution of MEPE between OHO and non-OHO human bone tissue (Fig. 6 and Table 3), or between osteoporosis and osteomalacia (Fig.7 and Table 4), so MEPE might not be

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