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Adiponectin Replacement Therapy Attenuates Myocardial Damage in Leptin-deficient Mice with Viral Myocarditis

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The effects of adiponectin replacement therapy on myocardial damage were studied in leptin-deficient (OB) mice with acute viral myocarditis. Encephalomyocarditis virus was injected intraperitoneally into OB and wild-type (WT) mice. One subgroup of OB mice received no intervention and another subgroup received daily adiponectin replacement, simultaneously with viral inoculation. Differences in heart weight, cardiac histological score, numbers of infiltrating or apoptotic cells in the myocardium and the immunoreactivity of adiponectin receptors in myocytes were determined. The reactivity of adiponectin receptor 1 in myocytes from OB mice on day 4 and day 8 after viral inoculation was significantly decreased compared with that in myocytes from WT mice; the OB mice also had elevated cardiac weights and severe inflammatory myocardial damage. Adiponectin replacement in OB mice inhibited the development of severe myocarditis by augmenting myocyte adiponectin receptor 1 reactivity. Exogenously administered adiponectin may inhibit the progression of viral myocarditis through binding to the adiponectin receptor 1 in leptin-deficient conditions.

Osteoclast-like cells express receptor activity modifying protein 2: application of laser capture microdissection

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Abstract

Receptor activity modifying proteins (RAMPs) act as receptor modulators that determine the ligand specificity of receptors for the calcitonin (CT) family. The purpose of this study was to analyze the expression of RAMPs in osteoclast-like cells using the laser capture microdissection (LCM) technique. Mouse bone marrow and spleen cells were co-cultured on a film designed for LCM. After 10 days, 250 osteoclast-like cells were captured using the LCM system. Total RNA from these cells was used to synthesize cDNA and RT-PCR analysis was performed. Osteoclast-like cells expressed CT receptor (CTR), CT receptor-like receptor (CRLR) and RAMP2, but did not express RAMP1 or RAMP3. These results indicated (1) that a pure population of osteoclast-like cells can be prepared by LCM and gene expression of this population can be analyzed by RT-PCR and (2) that RT-PCR shows that osteoclast-like cells express RAMP2, CTR and CRLR, suggesting the potential for adrenomedullin binding to osteoclast-like cells. This is the first report that osteoclast-like cells express RAMP2.

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Introduction

The calcitonin (CT) family of peptides comprises five known members: CT, amylin (AMY), two CT gene-related peptides (CGRP1 and CGRP2) and adrenomedullin (ADM). Receptor activity modifying proteins (RAMPs) comprise a family of accessory proteins for G protein-coupled receptors, three of which act as receptor modulators that determine the ligand specificity of receptors for CT family members. CT receptor-like receptor (CRLR) has been shown to form a high affinity receptor for CGRP, when associated with RAMP1, or, when associated with RAMP2 or RAMP3, to specifically bind ADM (McLatchie *et al.* 1998, Sexton *et al.* 2001). RAMPs are type I transmembrane proteins that share ~30% amino acid identity and a common predicted topology, with short cytoplasmic C termini, one transmembrane domain and large extracellular N termini that are responsible for the specificity (McLatchie *et al.* 1998, Fraser *et al.* 1999). More recently, CT receptor (CTR) was demonstrated to form heterodimeric complexes with RAMP. CTR/RAMP1 and CTR/RAMP3 heterodimers exhibited the pharmacological profiles of receptors specific for AMY (Christopoulos *et al.* 1999, Muff *et al.* 1999, Sexton *et al.* 2001).

There is significant interest in analyzing gene expression of distinct cell populations. Heterogeneous populations of cells within tissues of various types possess correspondingly different patterns of gene expression, and these cell types must be separated from one another for accurate assessment of gene expression. Tong *et al.* (1994) has reported that a microisolation system using a micromanupilator tool was applied for mRNA phenotyping of a blood cell lineage. Laser capture microdissection (LCM) is a particularly useful tool for recovering small cell samples and even enables the collection of individual cells from tissue sections (Emmert-Buck *et al.* 1996). This method facilitates the separation of histologically distinct cells so that proteins, DNA or RNA from these cells can be analyzed in isolation from the surrounding cells (Bonner *et al.* 1997). Osteoclasts act centrally in the remodeling of bone in normal and diseased states. Nonetheless, because of their low numbers within bone, cell culture model systems have been increasingly used to investigate the biochemical functions of osteoclasts (Udagawa *et al.*1989, Nakamura *et al.* 1998). However, because of their heterogeneity and adherence to the plate in such systems, there has been difficulty and controversy in analyzing these cell types. Thus, a more sensitive isolation method for osteoclasts is needed.

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To address this problem, we used LCM techniques to isolate a pure population of osteoclast-like cells. We then analyzed RAMP gene expression in microdissected osteoclast-like cells using RT-PCR.

Materials and methods

An *in vitro* osteoclast model

Osteoclast differentiation *in vitro* was induced using the technique described by Udagawa *et al.* (1989). Both bone marrow cells and spleen cells were obtained from 10- to 14-week-old male C57BL/6 mice (Charles River, Sagamihara, Japan). The bone marrow cells were collected from tibiae and femora. Splenic tissue was cut with scissors and dispersed by pipetting, then the spleen cells were collected by centrifugation at 1000 r.p.m. for 5 min at 4 °C. Bone marrow cells were co-cultured with spleen cells (2×10^6 cells/ml for each cell type) on a film produced for use in LCM (Matsunami Glass Co., Osaka, Japan) for 10 days at 37 °C in a humidified atmosphere of 5% CO_2. Cultures were fed with α-modified Eagle's medium supplemented with penicillin and streptomycin, 10% fetal calf serum (Hyclone, Logan, UT, USA) and 10^{-8} M $1,25(OH)_2$ D_3 (Calbiochem-Novabiochem Co., San Diego, CA, USA). Multinucleated osteoclast-like cells were then isolated using LCM. All the animal experimental procedures were approved by the Animal Care and Use Committee of Wakayama Medical University (Wakayama, Japan).

LCM of samples

Before LCM, cells were fixed in ethanol for 1 min and stained for 3 min with filtered hematoxylin. They were then washed with sterilized water and air-dried for 10 min. LCM of cultured osteoclast-like cells was performed using the Application Solutions Laser Microdissection System (Leica Microsystems Co., Tokyo, Japan) according to the manufacturer's instructions.

RNA isolation

Total RNA was extracted from 250 LCM-captured cells and 250 spleen cells. The spleen cells used for RNA extraction were from an aliquot of those prepared for the co-culture system. Total RNA extraction was performed using TRIzol LS Reagent (Invitrogen Life Technologies Co., Carlsbad, CA, USA) as described by the manufacturer. Briefly, 170 μl TRIzol reagent was added to a tube containing LCM cells and this was incubated for 5 min at room temperature. Forty microliters of chloroform were then added and the tube was incubated at room temperature for a further 15 min. The samples were then centrifuged at 12 000 ***g*** for 15 min. The aqueous phase was transferred to a new tube and isopropyl alcohol was added followed by centrifugation at 12 000 ***g*** for 10 min. The RNA precipitate was washed with 70% ethanol and dissolved in 20 μl sterilized water.

RT-PCR

The SUPERSCRIP One-Step RT-PCR with PLATINUM *Taq* (Invitrogen Life Technologies Co.) was used to synthesize cDNA and PCR was performed as described by the manufacturer. The nucleic acid sequences of primers used for RT-PCR are shown in Table 1. RT-PCR reactions were initially performed in a 25 μl reaction volume containing 1 μl of each primer (at 100 ng/μl) and 3 μl RNA as template. The reactions were run at 55 °C for 30 min (cDNA synthesis) and 94 °C for 2 min (pre-denaturation), followed by 45 cycles of 94 °C for 30 s (denaturation), 53 °C for 30 s (annealing) and 72 °C for 30 s (extension), followed by 7 min at 72 °C (final extension). To increase the detection capacity, we performed a second round of PCR. The second-round PCR reactions were carried out using Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT, USA) with 8 μl RT-PCR products as template (final 25 μl reaction mixture) under the following conditions: 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. In the second-round PCR, CTR was amplified using 2nd sense and anti-sense primers (Table 1). The primers of CRLR, RAMP1, 2, 3, alkaline phosphatase (ALP) and β-actin for the second-round PCR were the same primers as those used in the initial RT-PCR. The samples were electrophoresed in 3% agarose gels and stained with ethidium bromide.

Results and discussion

We have developed a rapid and precise method for the isolation of pure populations of osteoclast-like cells using LCM. Figure 1 illustrates two osteoclast-like cells before and after LCM. Two hundred and fifty cells with > three nuclei each were isolated. Total RNA was extracted and RT-PCR was used to analyze multiple gene expressions. Figure 2 shows the RT-PCR results for CTR, RAMP1, 2 and 3, CRLR and β-actin. The predicted sizes were clearly visualized by ethidium bromide staining. RT-PCR results showed that the ubiquitous gene, β-actin, was amplified from both spleen and osteoclast-like cells, whereas CTR mRNA was amplified from osteoclast-like cells alone. RAMP1 and RAMP3 mRNAs were amplified from spleen cells alone. RAMP2 and CRLR mRNAs were amplified from both types of cells.

In the present study, we were able to isolate a pure population of osteoclast-like cells and detect a series of gene expressions. Two hundred and fifty cells were

Table 1 *Oligonucleotide sequences used for PCR*

	Sequence	Size
Target		
CTR	1st sense, 5′-GTCTTGCAACTACTTCTGGATGC-3′	255 bp
	1st antisense, 5′-AAGAAGAAGTTGACCACCAGAGC-3′	(Inoue *et al.* 1999)
	2nd sense, 5′-GTCTTGCAACTACTTCTGGATGC-3′	104 bp
	2nd antisense, 5′-GAAGATAGTACCAGCGTAGGC-3′	(U18542*)
RAMP1	Sense, 5′-CACCATCTCTTCATGGTCACTG-3′	187 bp
	Antisense, 5′-CAATCGTGTGCGCCACGTGC-3′	(AJ250489)
RAMP2	Sense, 5′-TGGATCTCGGCTTGGTGTGAC-3′	217 bp
	Antisense, 5′-GCAAGGTAGGACATGTGTTTCG-3′	(AJ250490)
RAMP3	Sense, 5′-TTGTGGTGAGTGTGCCCAGG-3′	189 bp
	Antisense, 5′-CCCATGATGTTGGTCTCCATC-3′	(AJ250491)
CRLR	Sense, 5′-TGTAATAACAGCACGCATGAG-3′	225 bp
	Antisense, 5′-GTTATTGGCCACTGCCGTGA-3′	(AF146525)
ALP	Sense, 5′-ATCGGGACTGGTACTCGGATAA-3′	152 bp
	Antisense, 5′-ATCAGTTCTGTTCTTCGGGTAC-3′	(NM007431)
β-Actin	Sense, 5′-GTGGGCCGGTCTAGGCACCA-3′	246 bp
	Antisense, 5′-GGTTGGCCTTAGGGTTCAG-3′	(Flores-Delgado *et al.* 1998)

*Accession number of GeneBank.
ALP, alkaline phosphatase.

used for RNA extraction and cDNA synthesis. Three microliters of the 20 μl cDNA solution was successful for each gene amplification. Approximately 40 cells were therefore used for RT-PCR analysis. Naot *et al.* (2001) have reported that osteoclastic cells such as primary osteoblasts and UMR 106-06 cells expressed all three types of RAMP analyzed using RT-PCR. A very high expression of mRNA for RAMP2 was detected in those cells, compared with those for RAMP1 and RAMP3. Previous studies showed that osteoblast but not osteoclast cells express ALP (Tong *et al.* 1994). To exclude the possibility of osteoblast contamination, we investigated ALP mRNA expression in the microdissected osteoclast-like cells. The result showed that no ALP mRNA was detectable (Fig. 3), which supported the idea that RAMP2 was amplified from osteoclast-like cells. Thus, LCM is a useful technique for isolation of small cell samples, and our strategy might be extended to other procedures, such as quantitative RT-PCR to measure mRNA levels in the osteoclast. The bone marrow macrophages are the precursors of osteoclasts; it will be interesting to compare gene expression between osteoclasts and bone marrow macrophages. Immunostaining of the Fc receptor, C3 receptor or vitamin D receptor will help to distinguish those cell types in our co-culture system. However, in order to perform RNA

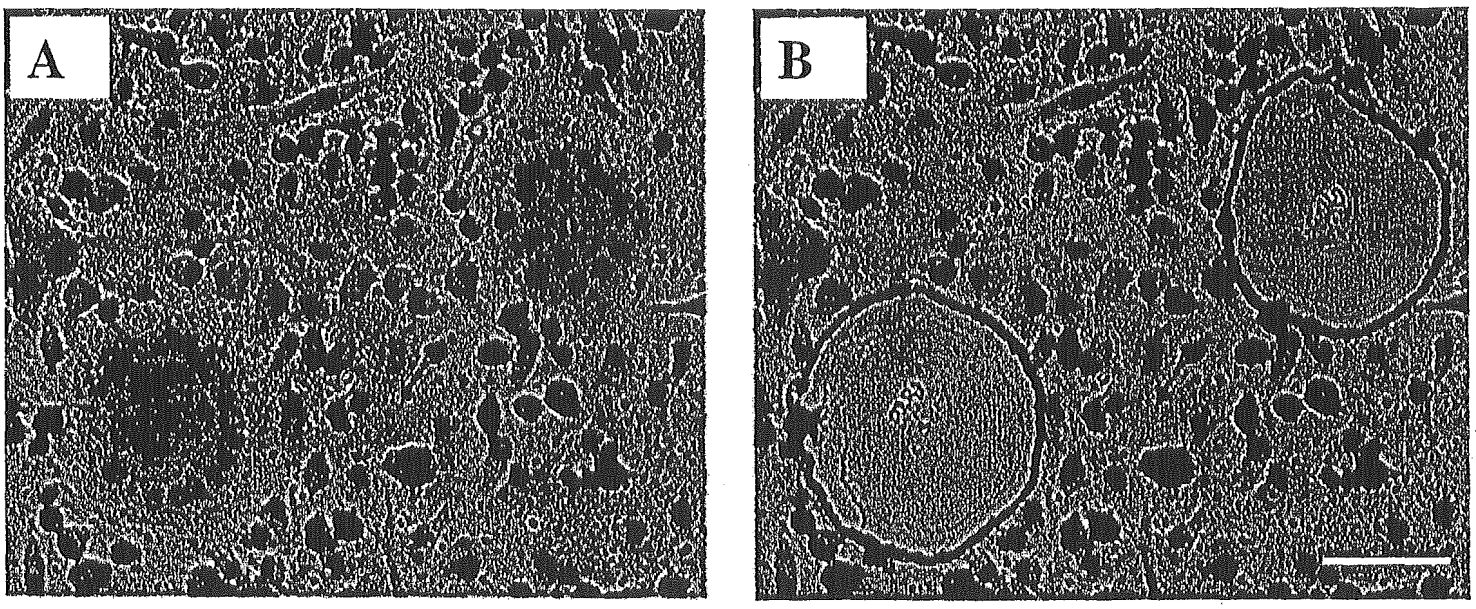

Figure 1 Osteoclast-like cells were isolated by using LCM. Two osteoclast-like cells (A) before and (B) after microdissection are shown. Stained by hematoxylin; bar denotes 50 μm.

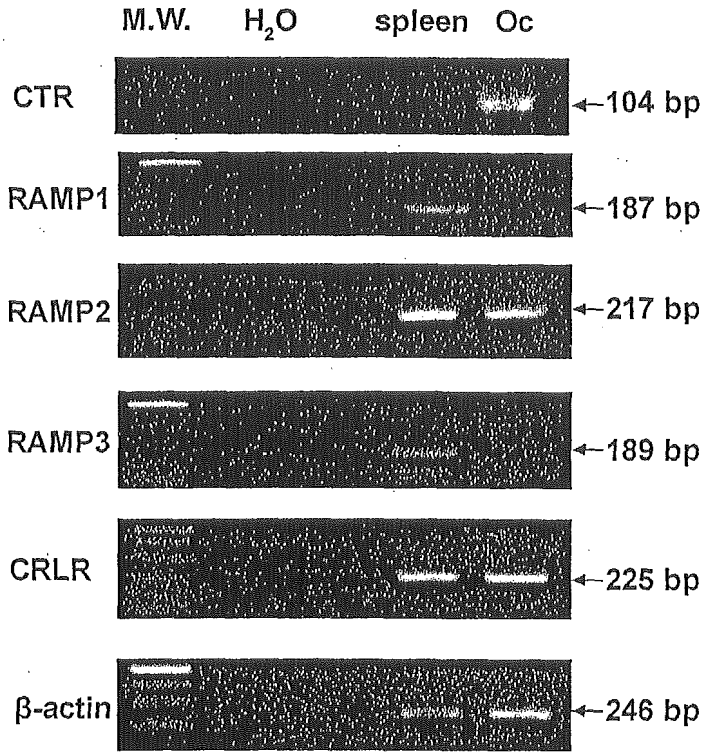

Figure 2 mRNA expression of various genes in spleen and osteoclast-like cells. Total RNA was extracted from microdissected osteoclast-like cells and spleen cells, RT-PCR was carried out to analyze the gene expression. β-actin served as the positive control. The products were electrophoresed in 3% agarose gels and stained with ethidium bromide. RAMP1, RAMP2, RAMP3 and CRLR were detected in spleen cells. M.W., molecular weight markers; H_2O, H_2O as template served as negative control; spleen, using RNA from spleen as template; Oc, using RNA from osteoclast-like cells from LCM as template.

analysis after immunostaining, further efforts should be made to modify the conventional staining protocol to protect RNA from degradation.

Our findings that osteoclast-like cells expressed RAMP2 and CRLR as well as CTR provide the first evidence that osteoclasts express RAMP2. These results suggest that osteoclasts may have the ability to bind

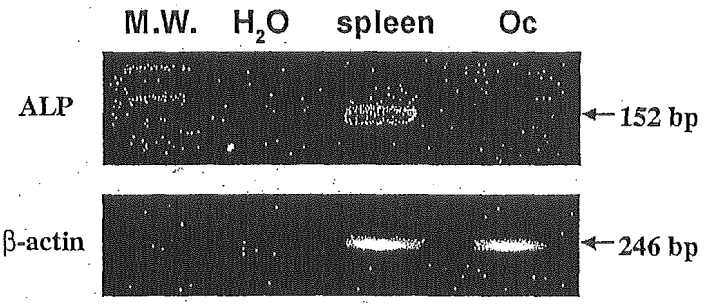

Figure 3 RT-PCR analysis of ALP expression. ALP expression was detected in spleen cells but not in osteoclast-like cells. M.W., molecular weight markers; H_2O, RT-PCR products using H_2O as template; spleen, using RNA from spleen as template; Oc, using RNA from osteoclast-like cells from LCM as template.

ADM through the CRLR/RAMP2 heterodimer. ADM is a 52 amino acid peptide first described in a human phaeochromocytoma but subsequently found to be present in many tissues, including the vascular system and bone tissue (Kitamura *et al.* 1993). Naot *et al.* (2001) has suggested that ADM is mitogenic to osteoblasts, raising the possiblity that ADM is a local regulator of bone growth; however, the action of ADM or RAMP on the osteoclast is not clear. It has been reported that bone abnormalities were observed in both CTR +/− and AMY +/− mice, thereby ruling out the possibility that AMY uses CTR to inhibit osteoclastogenesis *in vivo* (Dacquin *et al.* 2004).

In summary, we have demonstrated that LCM is a useful solution for osteoclast research. We found that osteoclast-like cells expressed mRNAs for CTR, CRLR and RAMP2 but not RAMP1 or RAMP3; RAMP2 may therefore play an important role in osteoclast function. Further study is needed to elucidate the role of RAMP2 and its relationship to the CT. family of receptors.

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Erratum

The authors apologize for an error in the paper by Li Chen, Jinsong Zhu, Guoqiang Sun and Alexander S Raikhel which appeared in the December 2004 issue of *Journal of Molecular Endocrinology* **33** 743–761, titled 'The early gene *Broad* is involved in the ecdysteroid hierarchy governing vitellogenesis of the mosquito *Aedes aegypti*'.

Page 757 read:
and JH blocks their appearance in larval *Manduca* (Zhou & Riddiford 2002).

When it should have read:
and JH blocks their appearance in larval *Manduca* (Zhou *et al.*, 1998).

Page 759 read:
misexpression of Z1 causes the reappearance of a pupa cuticle gene *Edg78E* and suppresses a larval cuticle gene *Lcp65A-b*.

When it should have read:
misexpression of Z1 causes the reappearance of a pupal cuticle gene *Edg78E* and suppresses a larval cuticle gene *Lcp65A-b* (Zhou & Riddiford, 2002).

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Original articles

Matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes in human bone

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Abstract 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 mineralization. We developed a rabbit polyclonal antibody directed against recombinant human MEPE (rhMEPE) after cloning its cDNA from the cDNA library of a nasal tumor tissue causing OHO. Using this antibody, we analyzed the distribution of MEPE in human bones by immunohistochemistry. In bone specimens from normal subjects, MEPE was predominantly expressed by osteocytes and not by osteoblasts. In bone specimens from patients with osteomalacia, however, MEPE was focally expressed by deeply located osteocytes. We also compared the MEPE positivity of osteocytes in mineralized bone and non-mineralized osteoid obtained from patients with osteomalacia and osteoporosis. Among osteomalacia patients, MEPE positivity was seen in 87.5 ± 8.6% of the osteocytes from mineralized bone compared with 7.8 ± 6.4% of those from osteoid. Among osteoporosis patients, MEPE positivity was found in 95.3 ± 0.5% of the osteocytes from mineralized bone compared with 4.9 ± 5.7% of those from osteoid. MEPE was mainly expressed by osteocytes embedded in the matrix of mineralized bone from patients with osteomalacia or osteoporosis. Our data provide the first histological evidence that MEPE is predominantly expressed by osteocytes in human bone, with significant expression by osteocytes within mineralized bone.

Key words MEPE · osteocyte · immunohistochemistry · mineralization · human

Introduction

Matrix extracellular phosphoglycoprotein (MEPE) is a glycosylated protein that was originally cloned from the tumors of patients with oncogenic hypophosphatemic osteomalacia (OHO) [1]. Because of its high expression in tumors that cause OHO, MEPE is regarded as a candidate phosphatonin, a putative humoral factor causing hypophosphatemic osteomalacia [2]. The MEPE gene has similarities with the genes of bone-tooth mineral matrix phosphoglycoproteins called SIBLINGs (small integrin-binding ligand with N-linked glycosylation) which contain RGD sequences that have been proposed as essential for integrin-receptor interactions [3]. This group of proteins includes osteopontin (OPN), dentin sialo phosphoprotein (DSPP), dentin matrix protein 1 (DMP1), and bone sialo protein (BSP).

MEPE also appears to be associated with the mineralization of bone. Petersen et al. [4] reported that osteoblast/osteocyte factor 45 (OF 45), which is identical to MEPE, was specifically expressed in bone tissue and that its expression was increased during matrix mineralization mediated by rat bone marrow-derived osteoblasts. They also showed that this protein was highly expressed by osteocytes embedded within the bone matrix. Argiro et al. [5] reported that murine MEPE mRNA was expressed by fully differentiated osteoblasts in vitro and that its expression was markedly increased during murine osteoblast-mediated matrix mineralization in normal and Hyp mice. Recently, Gowen et al. [6] reported that OF 45 knockout mice showed an increase in bone mass due to an increase in osteoblast numbers and activity. These findings suggest that MEPE may have a direct influence on bone metabolism, not only on renal phosphate handling but also on the mineralization of osteoid.

Although MEPE expression at the protein level has been demonstrated in mice [6] and rats [4], MEPE expression in human bone remains unproven. We developed a rabbit polyclonal antibody directed against recombinant human (rh)MEPE, which was obtained by the expression of MEPE cDNA in *Escherichia coli*. In

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this study, we demonstrated the expression of MEPE histologically in bone tissue from normal subjects, patients with several types of osteomalacia (OHO, Fanconi's syndrome, and vitamin D-deficient rickets), and patients with osteoporosis.

Subjects, materials, and methods

Preparation of polyclonal anti-MEPE antibody

We constructed a cDNA library from the nasal tumor of a patient with OHO [7], and cloned human MEPE cDNA using primers designed from the reported MEPE DNA sequence [1]. Then we obtained rhMEPE by expression in *E. coli* and developed a rabbit polyclonal antibody against rhMEPE. Briefly, a rabbit was immunized with rhMEPE (1 mg) in Freund's complete adjuvant (Wako, Osaka, Japan) injected at multiple subcutaneous sites on the back and intramuscularly into both thighs. After 2, 4, 6, and 8 weeks, the rabbit was given a half dose (0.5 mg) of rhMEPE in Freund's incomplete adjuvant (Wako). One week after the final booster injection, the rabbit was killed to obtain 70 ml of antiserum. The anti-serum had a titer of around 10^6–10^7 when assayed by enzyme immunoassay (EIA), using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Wako). The anti-serum (6 ml) was diluted twofold with MAPSII binding buffer (Bio-Rad Laboratories, Tokyo, Japan), and applied to a column of Protein A-Sepharose FF (1.6 × 5.0 cm, 10 ml; Amersham Biosciences, Tokyo, Japan). An IgG fraction was eluted from the column with MAPSII elution buffer (Bio-Rad Laboratories), followed by neutralization. After dialysis against phosphate-buffered saline (PBS), the IgG fraction was chromatographed on a column of MEPE-coupled NHS-Hitrap (1 ml, containing 3 mg of coupled rhMEPE; Amersham Biosciences). The specific antibody fraction was eluted with 0.5 M NaCl–0.1 M glycine-HCl (pH 2.7). After neutralization and dialysis against PBS, the affinity-purified anti-MEPE IgG fraction was stored at 4°C until use. Starting from 6 ml of anti-serum, 16 mg of rabbit polyclonal anti-MEPE antibody was obtained. To confirm the specificity of this anti-rhMEPE antibody, a Western blot was performed of a crude cell lysate of *E. coli* expressing rhMEPE, using anti-MEPE IgG and HRP-conjugated goat anti-rabbit IgG (Wako) as the primary and secondary antibodies, respectively. To confirm the detectability of rhMEPE expressed by a mammalian host, immunoblotting with the anti-MEPE antibody of the culture medium of rhMEPE-transfected Chinese hamster ovary (CHO) cells was also performed.

Clinical profile of the subjects

Samples of normal bone tissue were obtained intraoperatively from two patients: intact bone was obtained from a site away from a traumatic fracture of the tibia in a healthy 18-year-old man (NC1), and intact fibula was obtained from a 2-year-old boy who underwent below-knee amputation for fibrosarcoma of the tibia (NC2). Informed consent was obtained in both cases.

Iliac bone samples affected by osteomalacia (OM1-4; $n = 4$) and osteoporosis (OP1-4; $n = 4$) were obtained from patients who were diagnosed on the basis of bone mineral density, laboratory data, and iliac bone biopsy findings. Of the patients with osteomalacia, two had OHO (OM1, 2), one had Fanconi's syndrome (OM3), and one had vitamin D-deficient rickets (OM4). The clinical characteristics of all patients are summarized in Table 1.

Preparation of specimens

Paraffin sections

Decalcified paraffin sections were prepared for immunohistochemistry to detect MEPE. Bone samples from NC1, NC2, and OM1 were fixed in 4% paraformaldehyde (pH 7.4) at 4°C for 24 h, decalcified in 20% ethylenediamine tetraacetic acid (EDTA) (pH 7.4), dehydrated through an ethanol series, and finally embedded in paraffin. The specimens were then 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 features of each bone specimen.

Methylmethacrylate (MMA) sections

Sections were also prepared from undecalcified tissue to distinguish between the calcified and noncalcified areas. Iliac bone samples from the four osteomalacia patients (OM1-4) and the four osteoporosis patients (OP1-4) were fixed in 70% ethanol, prestained with Villanueva bone stain for 7 days, dehydrated through an ethanol and acetone series, and embedded in MMA, as described [8]. To distinguish noncalcified osteoid as Villanueva-positive areas, dry sections (5-μm-thick) were cut, using a Jung Supercut 2065 Microtome (Leica Microsystems, Heidelberg, Germany) equipped with a tungsten carbide knife. For immunohistochemistry, serial wet sections (5-μm-thick) were cut with the same machine 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°C.

Table 1. Characteristics of patients

Patient	Age (years)	Sex	Diagnosis	Bone histomorphometric data			Serum data				
				OS/BS (%)	O.Th (μm)	MAR (μm/day)	Calcium (8.4–10.0 mg/dl)	Phosphorous (2.9–4.8 mg/dl)	ALP (69–135 IU/l)	1,25$(OH)_2$vitD (20–60 pg/ml)	25(OH)vitD (10–55 pg/ml)
NC1	18	M	Tibial fracture	NA	NA	NA	NA	NA	NA	NA	NA
NC2	2	M	Amputation	NA	NA	NA	NA	NA	NA	NA	NA
OM1	53	F	Oncogenic osteomalacia	83.6	42.8	CND	8.5	1.8	356	12.5	NA
OM2	58	F	Oncogenic osteomalacia	80.1	38.0	CND	8.0	1.6	472	18.0	29
OM3	35	F	Fanconi's syndrome	90.2	50.0	CND	8.2	2.4	947	9.2	24
OM4	53	F	Vitamin D deficiency	97.3	117.5	CND	7.1	2.4	264	23.5	16
OP1	53	M	Osteoporosis	16.7	10.2	0.78	8.3	4.6	118	61.3	6
OP2	54	F	Osteoporosis	20.2	9.4	NM	8.9	4.6	101	61.7	21
OP3	65	M	Osteoporosis	12.1	13.1	0.66	9.8	3.6	147	67.4	25
OP4	45	F	Osteoporosis	20.2	9.4	0.61	9.4	4.9	99	66.0	NA

OS/BS, osteoid surface/bone surface; O.Th, osteoid thickness; MAR, mineral apposition rate; CND, calculation not done; NM, no measurement; NA, not available; NC, normal control; OM, osteomalacia; OP, osteoporosis

Immunohistochemical staining

Paraffin-embedded tissue sections were deparaffinized, and MMA-embedded sections were deacrylated twice in acetone, for 8 min each time, and then decalcified in 20% EDTA (pH 7.4) for 1 h. After being rinsed 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 the sections were incubated in 10% normal goat serum to minimize nonspecific background staining. Next, the rabbit polyclonal antibody directed against human MEPE was applied to each section, followed by incubation overnight at 4°C. An isotype-matched IgG was used for control staining. Detection was then performed using the streptavidin biotin-peroxidase complex technique (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan) before the sections were developed in 3,3′-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) and counterstained with hematoxylin.

Analysis of MEPE expression by osteocytes

MEPE expression by osteocytes was analyzed in mineralized bone and non-mineralized osteoid from all bone samples. Sections immunostained for MEPE, as well as Villanueva-stained MMA-embedded sections, were examined under a light microscope (ECLIPSE E1000; Nikon, Tokyo, Japan). Five randomly chosen visual fields within the trabecular bone area were examined at 200× magnification. The number of osteocytes was counted in the mineralized and non-mineralized areas of the Villanueva-stained section. Then the number of MEPE-positive cells was counted in the immunostained serial section in the areas that corresponded to those characterized as mineralized or non-mineralized by Villanueva staining. Subsequently, the ratio of MEPE-positive osteocytes to the total number of osteocytes in the mineralized bone and the non-mineralized osteoid was calculated. Values for results are presented as means ± SDs. Statistical analysis was performed using the Mann-Whitney U-test, and statistical significance was established at the $P < 0.05$ level.

Results

Western blotting of rhMEPE using the anti-MEPE polyclonal antibody

The specificity of the anti-MEPE antibody was examined by the Western blotting of both a crude lysate of *E. coli* expressing rhMEPE and of the culture medium of CHO cells transfected with a MEPE expression plasmid (Fig. 1). When the *E. coli* lysate was tested, rhMEPE

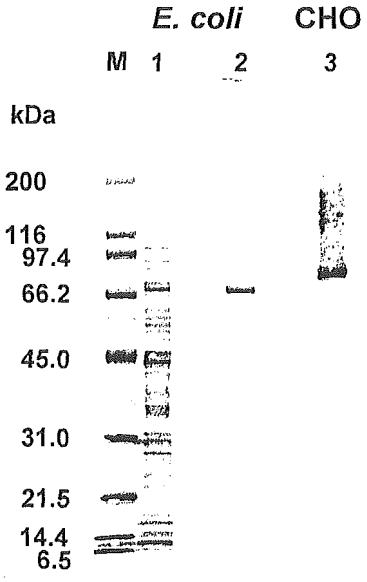

Fig. 1. Western blot analysis of recombinant human matrix extracellular phosphoglycoprotein (rhMEPE) expressed in *Escherichia coli* and Chinese hamster ovary (*CHO*) cells. A cell lysate prepared from *E. coli* expressing rhMEPE was stained with Coomassie Blue (*lane M*, molecular weight markers; *lane 1*, the *E. coli* lysate) and the lysate was also immunostained (*lane 2*) with anti-MEPE antibody. Culture medium from CHO cells transiently expressing rhMEPE was also analyzed by Western blotting (*lane 3*)

immunoreactivity was visualized as a single band at 67 kDa (lane 2; Fig. 1), demonstrating high specificity of the anti-MEPE antibody. When CHO cells were tested, a secreted protein of 70 kDa was selectively detected (lane 3; Fig. 1), confirming the specificity of the antibody. A broad, faint band was also observed at 100–150 kDa, indicating the secretion of heavily glycosylated forms of MEPE by the CHO transfectants.

Uniform MEPE expression in normal bone

MEPE expression was examined in normal bone tissue. Figure 2 shows MEPE expression in adult bone (NC1, tibial fracture), and Fig. 3 shows its expression in bone from a child (NC2, intact fibula). MEPE was strongly expressed by osteocytes in both cortical bone and trabecular bone (Fig. 2B, F), but it was not expressed by osteoblasts (Fig. 3C). Strong MEPE expression was observed in dendritic processes, as well as in the pericellular bone matrix of these bone-embedded osteocytes (Fig. 2D, H).

Focal MEPE expression in bone from osteomalacia patients

Bone specimens from osteomalacia patients were also examined. Figure 4 shows the bone specimen from a patient with OHO (OM1). MEPE expression was predominant in the osteocytes of cortical bone, although focal expression was also seen in trabecular bone (Fig. 4B); expression was more abundant in the central area

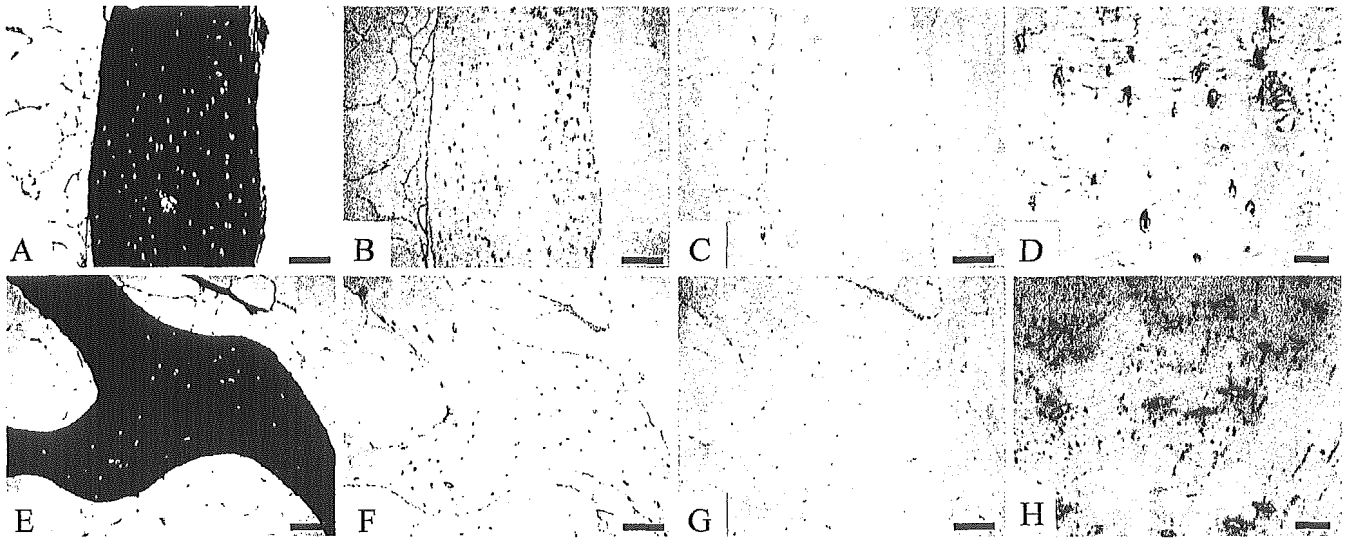

Fig. 2. Immunohistochemical detection of MEPE in normal human bone tissue (subject, NC1); **A–D** cortical bone; **E–H** trabecular bone from the tibia. **A,E** Staining with H&E. **B,D, F,H** Immunolocalization of MEPE; **C,G** negative control, and **D,H** higher magnifications of **B** and **F**, respectively. **B,F** MEPE is uniformly expressed by osteocytes embedded in the matrix of both cortical and trabecular bone. **D,H** The dendritic processes and pericellular bone matrix of osteocytes are also positive. *Bar*, 100 μm in **A–C** and **E–G**; *bar*, 20 μm in **D** and **H**

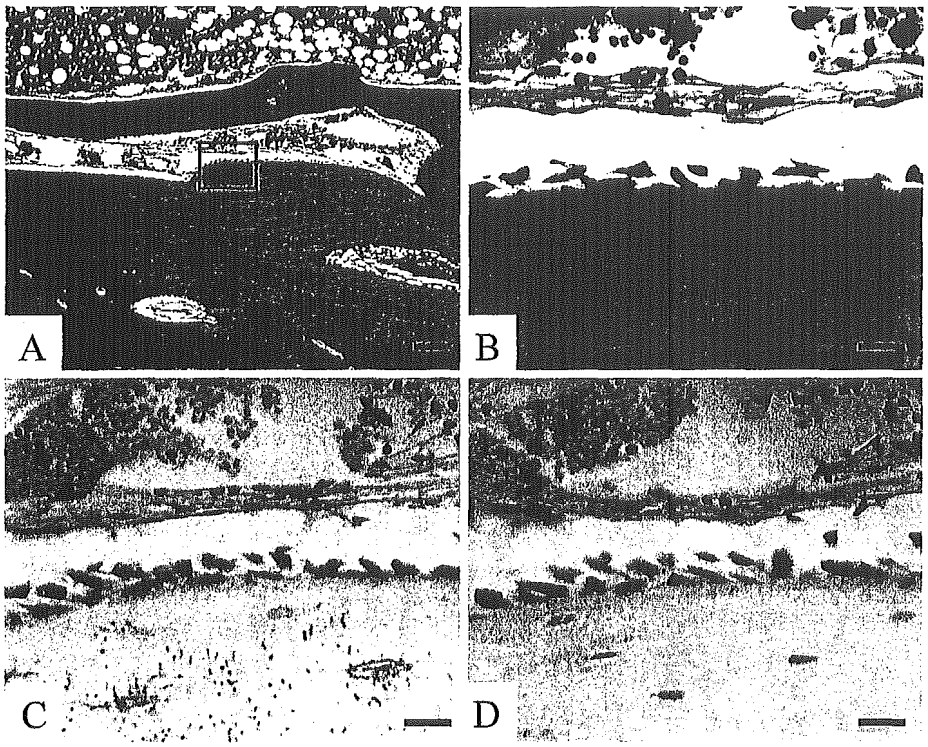

Fig. 3. Immunohistochemical detection of MEPE in immature bone (subject, NC2); **A, B** H&E staining; **C** anti-MEPE antibody; **D** negative control. **B** Higher magnification of the *boxed area in A*. **C** Osteoblasts are not stained, but osteocytes, pericellular bone matrix, and dendritic processes are stained, as was the case in mature bone (Fig. 2). *Bar*, 150 μm in **A**; *bar*, 20 μm in **B–D**

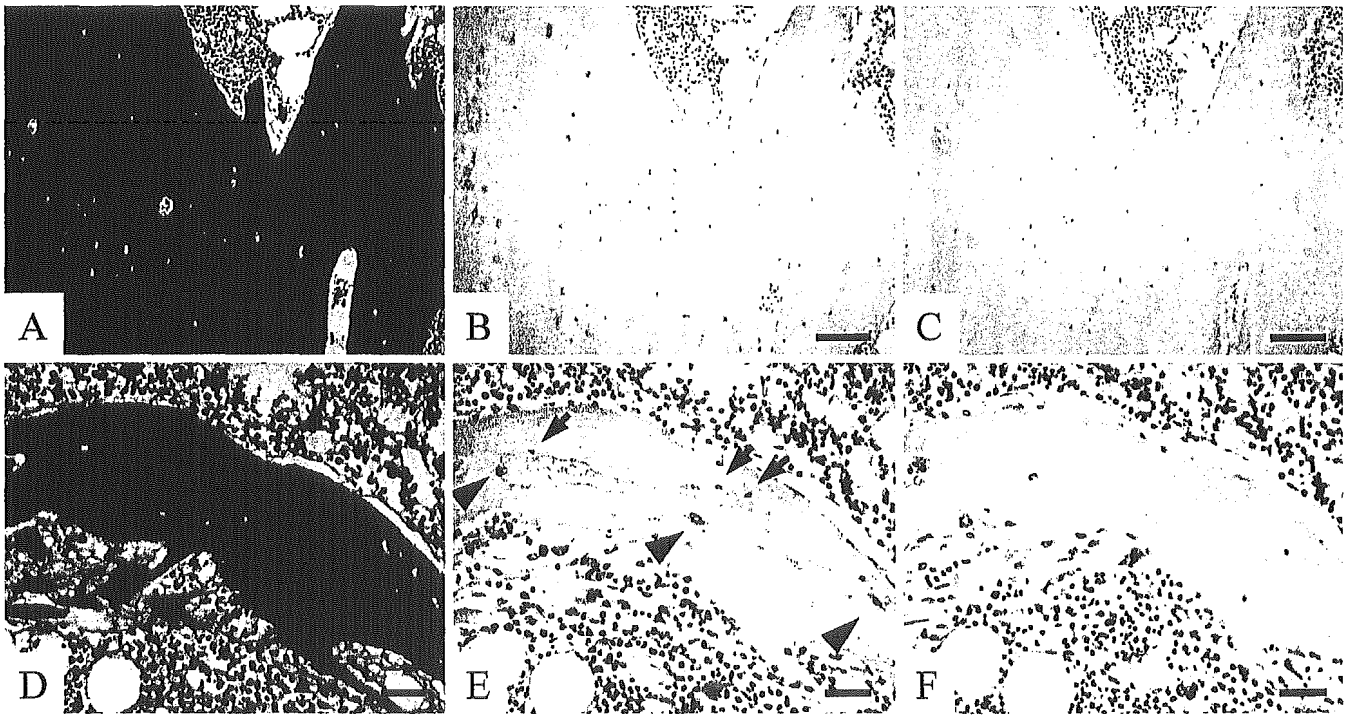

Fig. 4. Immunolocalization of MEPE in bone tissue from a patient with osteomalacia (OM1); **A,D** H&E; **B,E** anti-MEPE antibody; **C,F** negative control. **B** MEPE is expressed heterogeneously by osteocytes, and there is more positive staining in cortical bone (*CB*) than in trabecular bone (*TB*). **E** In trabecular bone, MEPE-positive cells are more abundant in the central area (*arrowheads*) than at the boundary zone (*arrows*). *Bar*, 100 μm in **A–C**; *bar*, 40 μm in **D–F**

of bone than at the boundary zone, which could be regarded as osteoid (Fig. 4E).

Abundant MEPE expression in mineralized bone in patients with osteomalacia and osteoporosis

To assess the expression of MEPE in osteoid, we performed Villanueva staining and MEPE immunostaining of bone specimens from osteomalacia patients, using serial sections. In the specimen from osteomalacia patient 1 (OM1), Villanueva staining revealed prominent osteoid at the boundaries of trabecular bone (Fig. 5A, B), and MEPE expression was mainly observed in the Villanueva-unstained mineralized bone area (Fig. 5C). In the other three osteomalacia patients, similar findings were obtained. In order to make a comparison with the pattern of MEPE localization in patients with osteoporosis, iliac bone specimens from the four osteoporosis patients were examined by the same method (Fig. 6). MEPE-positive osteocytes were mainly observed in the mineralized bone area (Fig. 6C), as was the case in the osteomalacia patients. All four osteoporosis patients showed a similar pattern of MEPE expression.

The MEPE-positive osteocyte ratio was calculated separately for mineralized bone and non-mineralized osteoid. In mineralized bone from the osteomalacia and osteoporosis patients, MEPE positivity was seen in 87.5 $\pm$ 8.6% and 95.3 $\pm$ 0.5% of the osteocytes, respectively (Table 2). In contrast, in the osteocytes in the non-mineralized osteoid of osteomalacia patients, MEPE positivity was shown in 7.8 $\pm$ 6.4%, while in the osteocytes in osteoid from osteoporosis patients MEPE positivity was shown in 4.9 $\pm$ 5.7% (Table 2). These findings show that there is significantly increased MEPE expression in the osteocytes within mineralized bone ($^*P < 0.05$) in both osteomalacia and osteoporosis patients, and that there are no significant differences in MEPE expression between these two metabolic bone diseases.

Discussion

In the present study, we developed a rabbit polyclonal antibody that targeted rhMEPE expressed in *E. coli* and then used it to prepare an affinity-purified anti-MEPE antibody. Specific detection of crude rhMEPE from *E. coli* and CHO cells by Western blotting with the anti-MEPE antibody (Fig. 1) demonstrated its high specificity for MEPE. The difference in the molecular weight of MEPE obtained from *E. coli* and CHO transformants suggested that glycosylation of this molecule occurred in CHO cells.

Using this antibody, we investigated the distribution of MEPE in normal human bone by immunohistochemistry and recognized its predominant expression in bone-embedded osteocytes, a finding compatible with the results already obtained in mice [6] and rats [4]. Furthermore, MEPE expression was observed in the dendritic processes and pericellular bone matrix of the osteocytes, but not in osteoblasts.

Previous in vitro studies have suggested a correlation between MEPE expression and bone mineralization. Petersen et al. [4] 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. [5] reported a correlation between MEPE expression and bone mineralization after the addition of glycerophosphate to osteoblast culture medium. We found abundant MEPE expression by osteocytes in mineralized bone matrix, but more limited expression in non-mineralized osteoid (Figs. 5, 6), using a previously reported combination of histomorphometry and immunohistochemistry [8]. In normal bone, the non-mineralized osteoid area is too small to allow the detection of osteocytes in the non-mineralized matrix by standard histological examination. This may account for the fact that previous immunohistochemical studies of normal rodent bone did not detect osteocytes without MEPE expression. Our results, combined with previous in vitro data, suggest that MEPE is strongly expressed during the mineralization of bone as osteoblasts undergo maturation into osteocytes. However, it is not clear whether MEPE expression precedes the onset of mineralization of the bone matrix or whether it is preceded by mineralization.

Osteocytes are easily defined by their location and typical stellate morphology, and they have a relatively small number of organelles, which are necessary for the production and secretion of bone matrix [9,10]. Several non-collagenous matrix proteins have been found in and around osteoblasts and osteocytes, including OPN [11–16], osteocalcin [17–19], BSP [20–22], biglycan [23–25], osteonectin [26], and DMP1 [27–29]. These proteins are thought to play various roles in promoting bone mineralization [30] and in facilitating the attachment of osteocytes to the bone matrix [31], and genetic studies with knockout animal models have supported such hypotheses [32–36]. Some of these proteins belong to the SIBLING family, sharing many characteristic motifs and structural features (such as the RGD motif and ASARM motif encoded on chromosome 4q), and are considered to have similar functions [3]. Because most of these proteins are expressed by both osteocytes and osteoblasts, few osteocyte-specific markers have been established, apart from DMP1 [29] and several monoclonal antibodies directed against avian osteocytes (mAb OB7.3 [37], mAb OB37.11 [38], and mAb SB5 [39]). Recently, mAb OB7.3 was shown to target

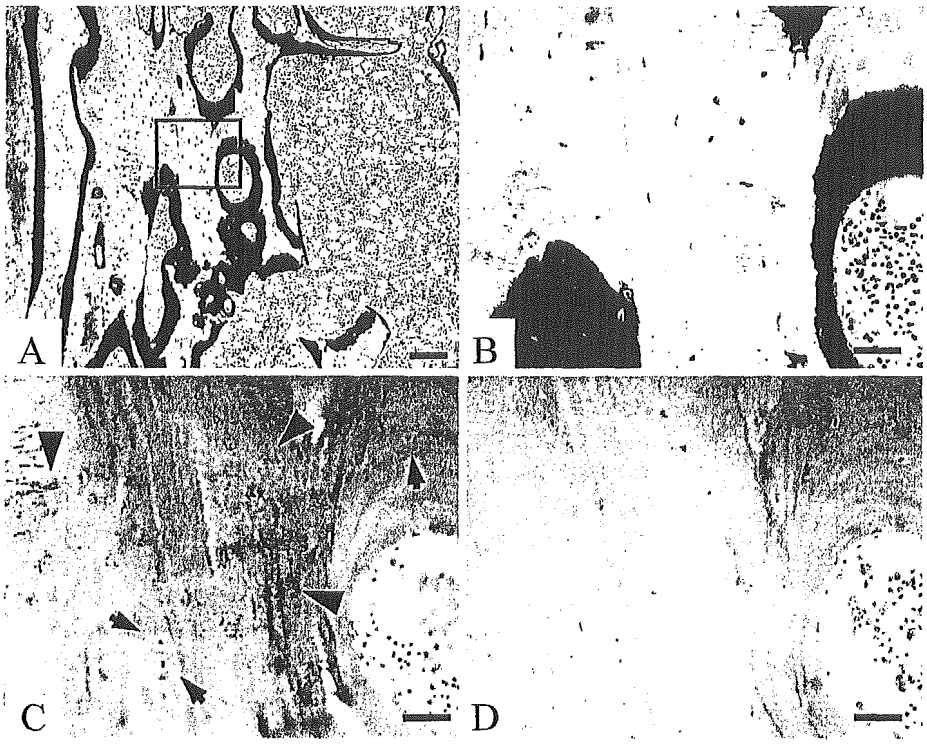

Fig. 5. Immunolocalization of MEPE in osteocytes of iliac bone tissue from a patient with oncogenic hypophosphatemic osteomalacia (OHO; OM1); **A,B** Villanueva bone stain; **C** anti-MEPE antibody; **D** negative control. **B** Higher magnification of the *boxed area in A*. **A** Abundant Villanueva-positive osteoid area is observed. **C** MEPE is expressed by osteocytes within the Villanueva-unstained mineralized bone area (*arrowheads*) and is not expressed by osteocytes within the osteoid (*arrows*). *Bar*, 150 μm in **A**; *bar*, 40 μm in **B–D**

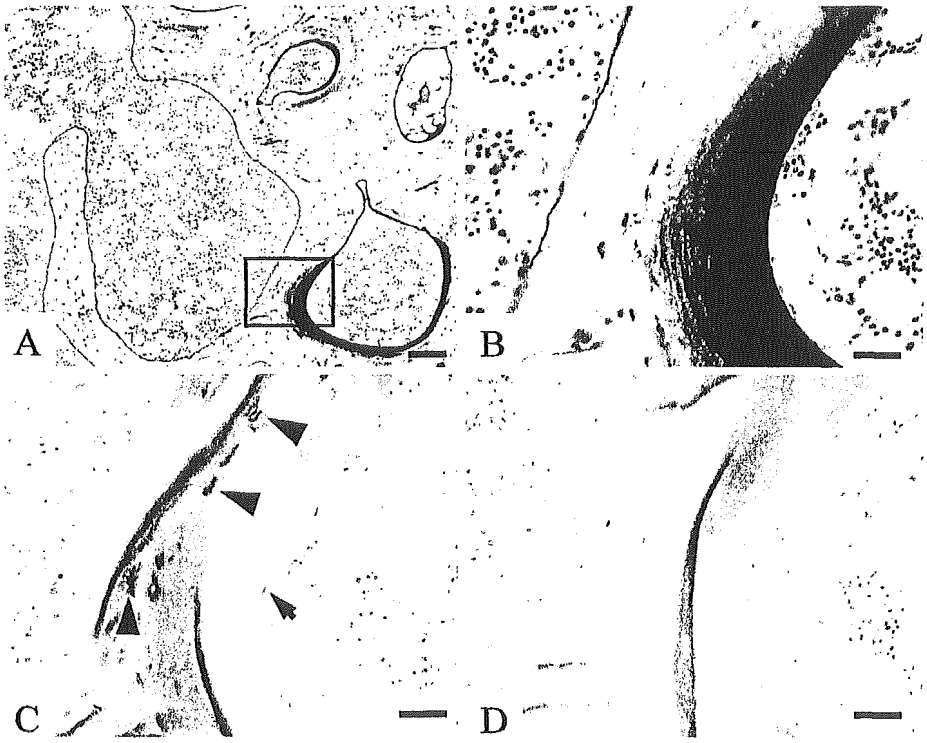

Fig. 6. Immunolocalization of MEPE in osteocytes of iliac bone tissue from a patient with osteoporosis (OP1); **A,B** Villanueva bone stain; **C** anti-MEPE antibody; **D** negative control. **B** Higher magnification of the *boxed area in A*. **A** The Villanueva-positive osteoid area is very small. **C** An osteocyte within the osteoid does not express MEPE (*arrow*), but osteocytes within mineralized bone express MEPE (*arrowheads*), as was the case in osteomalacia bone (Fig. 5). *Bar*, 150 μm in **A**; *bar*, 40 μm in **B–D**

the Phex (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) protein [40]. MEPE has already been shown immunohistochemically to be expressed by rodent osteocytes, and we also demonstrated here that it is expressed predominantly in human osteocytes, but not in osteoblasts. These findings raise the possibility that MEPE is one of the specific markers for osteocytes.

We were unable to detect a difference in the distribution of MEPE between OHO and non-OHO bone

Table 2. Number of matrix extracellular phosphoglycoprotein (MEPE)-positive osteocytes and MEPE positivity in bone tissue of osteomalacia and osteoporosis patients

	Mineralized bone			Osteoid		
Patient	Total OCY	MEPE(+) OCY	Positivity (%)	Total OCY	MEPE(+) OCY	Positivity (%)
OM1	88	66	75.0	52	9	17.3
OM2	223	211	94.6	104	5	4.8
OM3	191	173	90.6	74	3	4.1
OM4	127	114	89.8	83	4	4.8
Mean ± SD value			87.5 ± 8.6			7.8 ± 6.4*
OP1	156	148	94.9	6	0	0
OP2	147	121	96.0	9	1	11.1
OP3	61	58	95.1	6	0	0
OP4	164	156	95.1	12	1	8.3
Mean ± SD value			95.3 ± 0.5			4.9 ± 5.7*

Number of osteocytes is the total value in five examined fields
OCY, number of osteocytes; MEPE(+) OCY, number of MEPE-positive osteocytes; positivity, the ratio of MEPE-positive osteocytes/total osteocytes
Asterisk indicates significant difference between positivity in mineralized bone and in osteoid, calculated by Mann-Whitney *U*-test (*$P < 0.05$)

(Table 2), or between osteoporosis and osteomalacia (Figs. 5 and 6, Table 2), so it remains unclear whether MEPE has a direct effect on bone metabolism in patients with osteomalacia. Further investigations are therefore needed to assess the influence of MEPE on bone metabolism in osteomalacia.

In summary, we demonstrated MEPE expression by osteocytes in human bone histologically, using a new rabbit polyclonal anti-rhMEPE antibody. We also investigated MEPE expression in bone from patients with osteomalacia and osteoporosis, and found that it was abundant in osteocytes within mineralized bone in both these diseases.

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Title:MEPE proteins is highly expressed in osteocytes in human bone

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Abbreviated title: MEPE is expressed by osteocytes in human bone

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Abstract

We perfomed cDNA cloning of human MEPE from the cDNA library of a human nasal tumor tissue causing oncogenic hypophosphatemic osteomalacia (OHO). We obtained recombinant human MEPE protein (rhMEPE) and developed a rabbit polyclonal antibody against rhMEPE. Then we analyzed the distrbution of MEPE protein in human bones by immunohistochemistry. In the bone specimen from normal people, MEPE protein was predominantly expressed by osteocytes, but not by osteoblasts or bone-lining cells. In the bone specimen from osteomalacia patients, however, MEPE was focally expressed by deeply-located osteocytes. Then we compared the MEPE positivity of osteocytes between mineralized bone tissue and non-mineralized osteoid obtaines from osteomalacia and osteoporosis patients. In osteomalacia patients, MEPE positivity was seen in 87.5±8.6% of osteocytes in mineralized bone and only 7.8±6.4% in osteoid. In osteoporosis patients, MEPE positivity was found in 95.3±0.5% of osteocytes in mineralized bone versus 4.9± 5.7% in osteoid. MEPE protein was mainly localized in osteocytes embedded in the matrix of mineralized bone from both osteomalacia and osteoporosis patients. Our data provide the first evidence that MEPE protein is predominantly expressed by osteocytes in human bone tissue and that MEPE is predominantly localized to mineralized bone.

INTRODUCTION

Osteomalacia is a disorder of bone metablism that is generally characterized as a disturbance of mineralization with an increase of non-mineralized osteoid. Patients frequently present with symptoms of bone pain and muscle weakness (1). Hypophosphatemia and/or lack of vitamin D activity can cause this disordr, and the former state is thought to result from excessive renal phosphate leakage(1). Recently, abnormal renal phosphate handling has been associated with some uncharacterized phosphaturic factors, which are known as phosphatonins (2-3).

Matrix extracellular phosphoglycoprotein (MEPE) is a novel glycosylated protein cloned from the tumors of patients with oncogenic hypophosphatemic osteomalacia (OHO), and is regarded as a candidate phosphatonin (4). MEPE has been reported to be one of most highly expressed genes in OHO tumor (5). More recently, the same group demonstrated that MEPE can function as a phosphaturic factor (6).

MEPE also seems to be associated with bone mineralization (7-8). Argiro et al. showed that murine MEPE mRNA is expressed by fully differentiated osteoblasts in vitro and is down-regulated by vitamin D (7). They also demonstrated that its expression was markedly increased during murine osteoblast-mediated matrix mineralization, especially in Hyp mice (7). Petersen et al. reported that OF 45 (a rat homologue of MEPE) was specifically expressed in bone tissue and that its expression was increased during matrix mineralization mediated by rat bone marrow-derived osteoblasts. They also showed that the potein was highly expressed within osteocytes that are embedded within bone matrix (8). Recently, Gowen et al. reported that OF 45 knockout mice exhibit increase bone mass due to an increase of osteoblast numbers and activity (9).

The MEPE gene has similarities with the genes of bone-tooth mineral matrix phosphoglycoproteins, i.e., both contain RGD sequence motifs that are proposed to be essential for integrin-receptor interactions, called SIBLIGs (small integrin-binding ligand with N-linked glycosylation) (10). Osteopontin (OPN), dentin sialo phosphoprotein (DSPP), dentin matrix protein 1 (DMP1) and bone sialo protein (BSP) are included in this group (4) and are considered to have a similar function.

These findings suggest that MEPE may have a direct impact on bone metabolism,

not only on renal phosphate handling, but also on the mineralization of osteoid. Although its expression at the protein level has been established in mice and rats (8-9), its expression in human bone remains unproved. We developed a rabbit polyclonal antibody directed against recombinant human MEPE (rhMEPE), which was obtained by expression of its cDNA in *E. coli*. In this study, we demonstrated the expression of MEPE at the protein level in human bone tissue from normal persons, patients with several types of osteomalacia (OHO, Fanconi's syndrome, and vitamin D deficient rickets), and patients with osteoporosis.

MATERIALS AND METHODS

Cloning of MEPE cDNA from a nasal tumor tissue of an OHO patient

Total RNA was extracted from a nasal tumor tissue of an OHO patient (11) according to standard procedures, and poly(A)$^+$RNA was purified using oligo(dT)$_{30}$-latex suspension (TaKaRa Bio, Ohtsu, Japan). The cDNA was synthesized using a cDNA synthesis kit (TaKaR Bio). The cDNA was ligated into the *Eco*R I and *Xho* I sites of λ ZAPII vector(STRATAGENE,La Jolla,CA)and packaged into phage λ with an *in vitro* packaging kit (SRATAGENE). The phage λ was infected into *E. coli* XL1-Blue MRF' strain for amplification With the constructed cDNA library as a template, a cDNA fragment of human MEPE was amplified by PCR using a forward primer ('5-GGAAACAGCTATGACCATG-3') derived from M13 reverse primer sequence in λ ZAPII vector and a reverse primer ('5-TCAGGTGCTCTCCTCTACATCAACTCACA-3')located in 3'untranslated region of MEPE cDNA (4). The PCR-amplified fragment was subcloned in pCR-Blunt vector (Invitrogen, Tokyo, Japan) to obtain pCR-MEPE-9. The nucleotide sequence of the MEPE cDNA thus obtained was confirmed using a 377 DNA sequencer (Applied Biosystems, Foster City, CA).