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Mineralized tissue cells are a principal source of FGF23

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Abstract

While fibroblast growth factor (FGF) 23 is known as a phosphaturic factor in inherited and/or acquired hypophosphatemic disorders, it also serves an endocrine role in normal phosphate homeostasis. FGF23 acts negatively on the NaPi2a cotransporter and 25-hydroxy D_3-1α-hydroxylase with a resultant decrease in renal phosphate (Pi) reabsorption, while osteoblasts appear to be a primary source of FGF23 whose expression is counter-upregulated by 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Here we have shown the distribution of FGF23 in normal rat bone and tooth, and its expression profile in fetal rat calvaria (RC) cell cultures. FGF23 mRNA was detectable in multiple fetal and adult tissues but levels were much higher in adult calvaria, femur and incisor, compared to the other tissues tested. Immunoreactive FGF23 was predominantly localized to osteoblasts, cementoblasts, and odontoblasts, with sporadic labeling in some chondrocytes, osteocytes and cementocytes. Notably, osteoclasts were also found to be a possible source of FGF23. Fetal bone and tooth germ cells labeled much less intensely than young adult osteoblasts and odontoblasts. In the RC cell model, FGF23 was expressed during osteoblast development. During matrix mineralization induced by β-glycerophosphate (βGP), FGF23 expression was transiently upregulated and then decreased to levels lower than in their non-βGP-treated counterparts. $1,25(OH)_2D_3$ markedly increased FGF23 expression concomitant with the inhibition of βGP-induced mineralization. Our data suggest that FGF23 expression in bone is closely correlated with bone formation *in vitro* and *vivo*, and points towards an important role(s) for FGF23 in young adult but not fetal mineralized tissues as a systemic factor for Pi homeostasis.

Keywords: FGF23 expression; Normal osteoblast; Bone cells; Mineralized tissue; Cellular development; Tooth

Introduction

The discovery of fibroblast growth factor (FGF) 23, the 22nd member of the FGF family, has provided significant new understanding of regulation of systemic Pi homeostasis [1]. FGF23 was identified originally as a phosphaturic factor in autosomal dominant hypophosphatemic rickets (ADHR) [2] and thereafter in tumor-induced osteomalacia (TIO) [3], renal phosphate wasting in McCune–Albright syndrome/fibrous dysplasia (FD) [4], familial tumoral calcinosis [5], and possibly X-linked hypophosphatemic rickets (XLH) [6]. FGF23 is essential for maintenance of phosphate homeostasis and/or 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) metabolism [3,7–13]. However, much remains to be understood about the mechanisms underlying FGF23 activities, as highlighted by the fact that not all the phenotypic characteristics of the FGF23-deficient mice seem consistent with those observed in FGF23

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overloading [3,8–11]. Notably, in spite of an increase in serum phosphate levels in *fgf23*$^{-/-}$ mice, hypomineralization of certain bones is seen [12,13]. An unexpected variance in serum FGF23 levels in humans has also been reported in hypophosphatemic disorders as well as normal conditions [14]. Elucidation of the cellular source of FGF23 and its regulatory mechanism(s) appear to be essential to address these apparent inconsistencies.

FGF23 mRNA was initially identified in a wide variety of tissues, including brain, thymus, thyroid, small intestine, testis, heart, liver and lymph node [2,3,15]. More recent real-time quantitative RT–PCR data suggest that FGF23 mRNA is most abundantly expressed in bone amongst tissues examined in normal mice [7]. Reports of FGF23 mRNA expression in osteoblastic cells are discrepant. No detectable expression was reported in primary bone cell cultures from mouse calvaria and limb bud cells, the mouse osteoblastic cell line, MC3T3-E1, or the human osteosarcoma cell line, SaOS2 [2]. However, immortalized mouse osteoblastic cells from the simian virus 40 (SV40) transgenic mice were found to express FGF23 mRNA but the levels did not change significantly during culture [7]. On the other hand, FGF23 mRNA expression was seen in SV40-transformed human fetal bone cells, and levels were up-regulated in relation to matrix mineralization and increased extracellular phosphate [16]. Although odontoblasts and cementoblasts share some features with osteoblasts, and dental defects are observed in some hypophosphatemic disorders [17], little information is available concerning whether FGF23 is also expressed during odontogenesis.

Levels of FGF23 mRNA are increased concomitant with mineralization defects in immortalized osteoblastic cells from SV40-transgenic Hyp (the homologue of XLH) mice, while interestingly SV40-transgenic Hyp osteogenic cells can differentiate normally [7]. FGF23 mRNA/protein is observed in osteoblasts and in newly formed osteocytes in healing fracture callus and fibrous dysplasia (FD) bone with a lower detection level in lining cells and osteocytes in normal conditions [4]. Moreover, Hyp mice crossed with FGF23-deficient mice are reported to be indistinguishable from FGF23-null mice, both in terms of serum phosphate levels and in skeletal phenotype [13]. Taken together, these observations suggest that expression levels of FGF23 are correlated with normal and pathophysiological bone formation and metabolism.

Recent data have implicated FGF23 as a counter-regulatory phosphaturic hormone for vitamin D. For example, 1,25 $(OH)_2D_3$ up-regulates FGF23 in bone (especially in osteoblastic cells, which express the $1,25(OH)_2D_3$ receptor) [18,19], but the phenotype of vitamin D receptor-null mice supports the lack of a direct effect of $1,25(OH)_2D_3$ on bone mineralization [20]. Thus, a novel vitamin D–FGF23 system appears to exist and be associated with bone mineralization, but details of expression and regulation of FGF23 during normal bone formation and metabolism are still fragmentary. We have therefore determined the distribution of FGF23 in normal rat bone and tooth formation *in vivo* and its expression profile during normal osteoblast development and matrix mineralization *in vitro*.

Materials and methods

Animals

Timed-pregnant or young adult (8 weeks old) male Wistar rats were housed and handled to minimize pain or discomfort to animals according to protocols approved by Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development, Hiroshima University. Rats for histological studies were anesthetized with an intraperitoneal injection of sodium pentobarbital just before sampling. To obtain calvaria cells (see below), both fetuses and their mothers were killed by cervical dislocation.

Tissue preparation and immunohistochemistry

Twenty-one-day-old fetal rat bones and lower jaws were immersed in 4% paraformaldehyde (PFA) in PBS at 4 °C overnight. Eight-week-old young adult male rats were perfused with 4% PFA in PBS, and bones and lower jaws were post-fixed with the same fixation solution at 4 °C overnight. These tissues were then decalcified with 20% EDTA in PBS (pH 7.4) at 4 °C, rinsed, and routinely embedded in paraffin. Fetal (E21) rat calvaria (RC) cell cultures were cultured for 14 days as described (see below) until bone nodules were forming, and were then fixed in 4% PFA in PBS at 4 °C for 1 h, rinsed and stored at −80 °C until use. Deparaffinized sections (5 μm in thickness) or culture dishes (subjected to two freeze–thaw cycles) were pretreated with Dako® Protein Block (DAKO, Carpinteria, CA) at room temperature (RT) for 1 h and incubated with goat anti-FGF23 antibody (1:200, Santa Cruz Biotech, Santa Cruz, CA) at 4 °C overnight, followed by incubation with biotinylated secondary antibody (1:200; Vector Lab, Burlingame, CA) for 1 h at RT. Sections were then treated with 0.3% H_2O_2 in methanol at RT for 30 min. All specimens were incubated with ABC reagents (1:100, Vector Lab) for 30 min at RT, followed by DynaChrome DAB Chromogen detection (IMMUNON, Pittsburgh, PA). The specimens were counterstained with hematoxylin or methylgreen. Each incubation step was followed by two washes with PBS (5 min each). As negative control, a large amount of blocking peptide specific to FGF23 (Santa Cruz Biotech) was used according to manufacturer's directions.

Cell culture

RC cells were isolated by sequential collagenase digestion (type I; Sigma-Aldrich, St. Louis, MO) from timed pregnant Wistar rats as described [21]. Cells from the last four of five digestion fractions were separately grown in αMEM supplemented with 10% fetal calf serum (FCS, Biological Industries, Kibbutz beit haemek, Israel) and antibiotics. After 24 h, cells were trypsinized, pooled, and grown in 35-mm dishes (0.3×10^4 cells/cm^2) in the same medium supplemented additionally with 50 μg/ml ascorbic acid (osteogenic medium). To induce matrix mineralization, 10 mM β-glycerophosphate (βGP) was added into cultures in combination with or without $1,25(OH)_2D_3$ (0.1–10 nM), when unmineralized osteoid-like nodules had developed (day 12, designated mature nodule cultures).

Osteoclast-rich populations were obtained from co-cultures of mouse bone marrow cells with osteoblastic cells [22]. Briefly, primary mouse osteoblastic cells were prepared from newborn ddY mouse calvariae as described above. Bone marrow cells (5×10^6 cells) from 8-week-old ddY mouse tibiae and femora were co-cultured with osteoblastic cells in αMEM supplemented with 10% FCS, 10 nM $1,25(OH)_2D_3$ and 1 μM prostaglandin E_2 in 100-mm dishes. Seven days after incubation, adherent cells were detached by 0.2% collagenase (Wako Pure Chemical, Osaka, Japan), replated for 10 h and then treated with PBS containing 0.001% actinase E (Kaken Pharmaceutical, Tokyo Japan) and 0.02% EDTA for 10 min at 37 °C to remove non-osteoclastic cells. Majority (ca. >90%) of cells remaining attached were osteoclasts. All cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2, and medium was changed every second or third day in general.

ALP/von Kossa staining

For cytochemical detection of alkaline phosphatase (ALP)-positive nodules comprising osteoblast populations, cultures were fixed in 10% neutral buffered