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The autosomal dominant hypophosphatemic rickets (ADHR) gene is a secreted polypeptide overexpressed by tumors that cause phosphate wasting

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ABSTRACT The gene mutated in autosomal dominant hypophosphatemic rickets (ADHR), a phosphate wasting disorder, has been identified as FGF-23, a protein that shares sequence homology with fibroblast growth factors (FGFs). Patients with ADHR display many of the clinical and laboratory characteristics that are observed in patients with oncogenic hypophosphatemic osteomalacia (OHO), a disorder thought to arise by the secretion of a phosphate wasting factor from different mesenchymal tumors. In the present studies, we therefore investigated whether FGF-23 is a secreted factor and whether it is abundantly expressed in OHO tumors. After transient transfection of OK-E, COS-7, and HEK293 cells with the plasmid encoding full-length FGF-23, all three cell lines efficiently secreted two protein species into the medium that were approximately 32 and 12 kDa upon SDS-PAGE and subsequent Western blot analysis using an affinity-purified polyclonal antibody to FGF-23. Furthermore, Northern blot analysis using total RNA from five different OHO tumors revealed extremely high levels of FGF-23 mRNA, and Western blot analysis of extracts from a sixth tumor detected the 32 kDa FGF-23 protein species. In summary, FGF-23, the gene mutated in ADHR, is a secreted protein and its mRNA is abundantly expressed by several different OHO tumors. Our findings indicate that FGF-23 may be a candidate phosphate wasting factor, previously designated "phosphatonin".

Introduction

Oncogenic hypophosphatemic osteomalacia (OHO) is a rare acquired disorder which is thought to arise from tumors that secrete a circulating phosphate wasting factor previously referred to as phosphatonin (1). Patients with OHO share many similarities with individuals affected by heritable disorders of phosphate wasting, such as autosomal dominant hypophosphatemic rickets (ADHR) and X-linked hypophosphatemia (XLH) and thus manifest hypophosphatemia, decreased or inappropriately normal serum 1,25 $(OH)_2$ vitamin D_3 concentrations, and osteomalacia (2-4). These clinical and laboratory findings resolve rapidly and completely if the entire tumor is removed surgically. Recently, we identified three different missense mutations affecting two amino acids in a novel gene, FGF-23 (GenBank Access. No. AF263536), that resulted in ADHR in four unrelated families (5). FGF-23 shares 25-35% homology with the family of fibroblast growth factors (FGFs) and is expressed at very low levels in normal tissues (5). The FGF-23 protein is predicted to comprise 251 amino acids, including a putative N-terminal signal peptide (residues 1-24) (5). Because OHO and ADHR share significant clinical and laboratory similarities, we sought to determine whether FGF-23 is indeed a secreted factor that is expressed in OHO tumors.

Materials and Methods

OHO tumors: Six different tumors from OHO patients were obtained upon surgical removal from: *Case a*) left thigh (6) (hemangiopericytoma); *b*) mandible (mixed connective tissue tumor) (7); *c*) left thigh (angiodysplasia) (8); *d*) sole of foot (hemangiopericytoma); *e*) nose (hemangiopericytoma); and *f*) distal femur (osteoblastic osteosarcoma). All patients presented with biochemical abnormalities characteristic of OHO, which resolved after tumor removal.

FGF-23 antibody production: Rabbit anti-human FGF-23 polyclonal antibodies were produced through standard protocols to the peptide CSQELPSAEDNSPMASD-COOH, which corresponds to residues 206-222 of human FGF-23 (Zymed Laboratories, Inc.; South San Francisco, CA). The antiserum was affinity purified against the peptide.

Bacterial FGF-23 synthesis: A human FGF-23 cDNA was produced by PCR amplification of RNA from human heart (Clontech Inc.; Palo Alto, CA) using Pfu polymerase (Gibco-BRL; Rockville, MD). An insert comprising nucleotides 73-756, that encode the full length FGF-23 without the predicted

signal peptide, was directionally cloned into the pQE30 vector in frame with an N-terminal 6xHis tag using the Type IV Qiaexpress Kit (Qiagen, Inc.; Valencia, CA). The plasmid, FGF23-6xHis pQE, was subsequently used for transformation of M15[pREP4] cells and IPTG was added to induce protein expression for 4h. FGF23-6xHis protein was purified by nickel chromatography minipreps as described by the manufacturer (Qiagen).

OHO tumor extract: Approximately 100 mg of tumor sample was resuspended in 0.5 ml of ice cold phosphate-buffered saline (PBS) supplemented with 75 μg/ml AEBSF protease inhibitor. The sample was homogenized for 30 sec on ice, then centrifuged at 1500 g and the cleared homogenate was used for further experiments. Protein concentrations were determined by Bradford protein assay (Bio-Rad, Inc.; Hercules, CA) with bovine serum albumin as the standard.

Western analysis: Protein samples and standards, for molecular mass determination, were electrophoresed on 15% SDS-PAGE mini-gels (Bio-Rad) and electroblotted onto nitrocellulose membranes. Membranes were incubated with 2.5 μg/ml anti-human FGF-23 antibody or mouse anti-pentaHis antibody, and subsequently with goat anti-rabbit or anti-mouse-HRP secondary antibody (1:1000) (Amersham, Inc; Piscataway, NJ), and visualized by enhanced chemiluminescence (ECL) (Amersham).

Cloning and expression of FGF-23: The FGF-23 open reading frame preceded by the Kozak sequence (bp –3 to 756) was amplified by RT-PCR from heart total RNA. This cDNA was then directionally inserted into the expression plasmid pcDNA3.1(+) (Invitrogen) to yield a construct designated pFGF23. The integrity of pFGF23 was confirmed by DNA sequencing.

Cell culture: HEK293, COS-7 (ATCC), and OK-E cells were cultured in DMEM-F12 (Gibco-BRL) with 10% fetal bovine serum (Hyclone; Logan, UT).

Transient transfection: Cells were seeded on 100 mm dishes and transiently transfected using the Fugene 6 reagent (Hoffmann-La Roche Inc.; Nutley, N.J.). After transfection, the incubation was continued for an additional 24 h. Conditioned media were concentrated 25 times with Centricon-10 columns (Millipore, Inc; Bedford, MA).

RNA isolation and Northern analysis: RNA was extracted from cells using a total RNA extraction kit (Qiagen) and from tumors using Trizol reagent (Life Technologies, Grand Island, NY). 10μg of total RNA was size fractionated on 1.2% agarose/formaldehyde gels. RNAs were capillary transferred to a nylon membrane (NEN-Dupont; Boston, MA) then hybridized at 42°C with an FGF-23 cDNA probe and washed under stringent conditions. All gels were stained with ethidium bromide to assure equal loading of RNA samples.

Results

Production of His-tagged human FGF-23 and polyclonal antibody assessment: To assess the specificity of the anti-FGF-23 polyclonal antibody, we produced recombinant human FGF-23 protein containing an N-terminal 6xHis tag instead of the putative signal peptide. The affinity-purified antibody recognized a protein of the predicted size of approximately 27 kDa in lysates from IPTG-induced bacteria, whereas no protein was detected in uninduced cultures (Fig. 1A). The same protein band was also detected with an anti-His antibody (not shown), indicating that both antibodies recognized identical proteins. Pre-immune sera failed to detect a protein in all experiments (not shown). These results confirm that the affinity-purified anti-FGF-23 antibody recognized recombinant human FGF-23 protein.

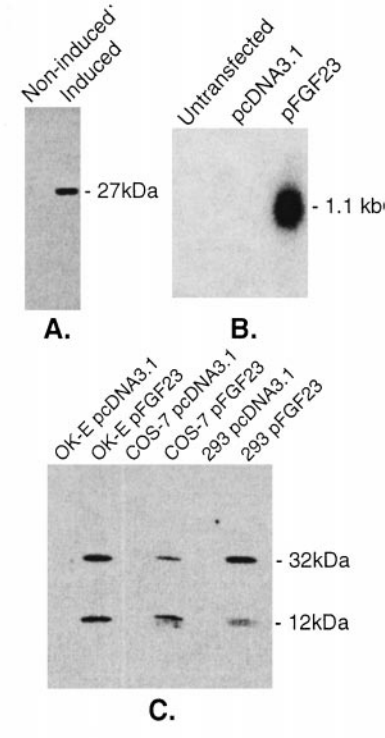

Fig. 1. Detection of FGF-23 protein and mRNA. A) Western blot analysis with the anti-FGF-23 antibody recognized a protein of 27 kDa from IPTG-induced bacteria transformed with FGF23-6xHis. B) Northern blot analysis using total RNA from HEK293 cells transfected with pFGF23 revealed a single transcript of 1.1 kb. C) Western blot analysis on concentrated conditioned media from cells transfected with pFGF23 revealed two protein bands of 32 and 12 kDa that reacted with the anti-FGF-23 antibody.

Human FGF-23 production by mammalian cells: OK-E, COS-7, and HEK293 cells were transiently transfected with pFGF23 or pcDNA3.1. Northern analysis of total RNA extracted after 24 h revealed a single mRNA species of 1.1 kb from all three cell lines that hybridized to the ^{32}P-labeled FGF-

23 cDNA probe (Fig. 1B, and not shown), whereas cells transfected with empty plasmid did not express an FGF-23 transcript. To determine if FGF-23 is a secreted protein, Western blot analysis was performed using conditioned media derived from the three transfected cell lines and the anti-FGF-23 antibody. Immunoreactive proteins of approximately 32 kDa and 12 kDa were detected in the conditioned media from the pFGF23-transfected cells, but not from pcDNA3.1 transfected cells (Fig. 1C). The 32 kDa band is the mature form of FGF23, and the 12 kDa species possibly represents a C-terminal degradation product of FGF23 because the smaller band is only detected after extended incubation of the cells in serum-free media. In addition, the secreted form of FGF23 is larger than its predicted size most likely due to core glycosylation, as tested by in vitro transcription and translation of pFGF23 in the presence of pancreatic microsomes (not shown). Taken together, these studies demonstrate that mammalian cells transiently expressing FGF-23 can generate an FGF-23 transcript and efficiently secrete the protein.

Assessment of OHO tumors for FGF-23 expression: To determine if OHO tumors express FGF-23 transcripts, we performed Northern analysis using total RNA isolated from five different tumors. The radiolabeled FGF-23 cDNA probe hybridized with FGF-23 transcripts of 3.0 and 1.3 kb, as well as a faint band at approximately 2.0 kb in all five tumors; in contrast the control RNAs from several other tissues demonstrated no hybridizing bands (Fig. 2A). To determine whether FGF-23 protein is present in OHO tumors, extracts from a sixth tumor were examined by Western blot analysis using the affinity purified anti-FGF-23 antibody. These studies revealed a protein of 32 kDa from the extracts (Fig. 2B). In summary, Northern and Western analyses indicate that FGF-23 is a secreted protein which is highly expressed by OHO tumors.

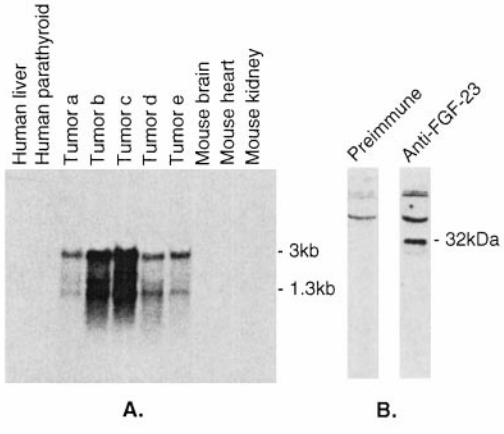

Fig. 2. OHO tumors express FGF-23. A) Northern blot analysis using total RNA from five different OHO tumors displayed strongly-hybridizing FGF-23 transcripts of 1.3 and 3.0 kb and a faint 2.0 kb band, after a 30 min exposure, whereas control tissues were negative. B) Western blot analysis of 2 μg of extract from another OHO tumor demonstrated an immunoreactive protein of 32 kDa that was not detected by pre-immune sera.

Discussion

We recently identified a novel gene, FGF-23, that is mutated in the renal phosphate wasting disorder ADHR (5). Our present studies demonstrate that FGF-23 is a secreted protein with a molecular mass of 32 kDa when expressed in OK-E, COS, and HEK293 cells, and when extracted from an OHO tumor (Figs. 1&2). Full-length FGF-23 is predicted to have a molecular mass of 25.3 kDa, excluding the signal peptide of 24 amino acids. Provided that the molecular mass is correctly estimated by SDS-PAGE, our findings could thus imply that FGF-23 undergoes post-translational modifications. Previous studies have estimated the molecular mass of partially purified OHO factors to be between 5-58 kDa (9-11). This may indicate that different tumors produce different phosphate wasting factors, or if FGF-23 is the only factor with these biological properties that is secreted from OHO tumors, the protein may undergo further modification during or after secretion.

FGF-23 mRNA is expressed at very low levels in a limited number of human and mouse tissues. RT-PCR is typically required to detect the transcript in tissues such as heart, liver, and thyroid/parathyroid. Northern blot analysis of poly A^+ RNA from non-OHO cancer cell lines required more than 7 days of exposure to visualize small amounts of FGF-23 mRNA (5). In contrast, OHO tumors express abundant amounts of FGF-23 mRNA, as indicated by the fact that only 30 min exposures were necessary to detect the transcript in 10 μg of total RNA, and FGF-23 protein is readily detectable by Western analysis using 2μg of OHO tumor extract (Fig. 2). Furthermore, this high level of expression is not limited to a single tumor since all investigated tumors, which arose in individuals from a variety of ethnic backgrounds, showed FGF-23 mRNAs after a brief exposure time of 30 min. Missense mutations were detected in the FGF-23 gene of our previously-studied ADHR families (5), therefore it is currently unknown whether these mutations lead to increased or diminished activity of the resulting mutant protein. Because only three mutations were found in four families in two amino acids, the disorder is rare, and the mutations occurred in a narrow region of the protein, we speculate that the ADHR mutations enhance the biological activity of FGF-23. It is possible that FGF-23 plays an important role in the normal regulation of phosphate homeostasis. Alternatively, it might affect phosphate homeostasis only when secreted in extremely abundant amounts by OHO tumors. This may be similar to the excessive synthesis of PTHrP, which leads only in the syndrome of humoral hypercalcemia of malignancy to abnormal regulation of calcium homeostasis (12). However, since X-linked hypophosphatemia (XLH) is caused by inactivating mutations

in the PHEX gene (13), it appears possible that FGF-23 could be a phosphate wasting hormone which is inactivated by PHEX. Consequently, inactivating mutations in PHEX, or a mutation in a putative PHEX cleavage site in FGF-23 could lead to increased circulating concentrations of FGF-23 which may then lead to renal phosphate wasting. It will be important in future experiments to measure serum levels of FGF-23 in ADHR, OHO, and XLH patients, and to compare the resulting findings with those in the murine XLH models. In summary, FGF-23 is a secreted factor that is abundantly expressed in six different OHO tumors and is therefore a candidate for the phosphate wasting factor previously designated as phosphatonin.

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