A mutation in the gene TNFRSF11B encoding osteoprotegerin causes an idiopathic hyperphosphatasia phenotype

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Idiopathic hyperphosphatasia is an autosomal recessive bone disease characterized by deformities of long bones, kyphosis and acetabular protrusion, increasing in severity as affected children pass through adolescence. Biochemical and histological evidence indicate that there is extremely rapid bone turnover, with indices of both bone resorption and formation greatly increased. A genome-wide search, in a family with three children affected by idiopathic hyperphosphatasia, suggested linkage to a locus on the long arm of chromosome 8 (8q24). The gene TNFRSF11B encoding osteoprotegerin (OPG), which lies within this locus, was an obvious candidate, given the critical role of OPG in regulating osteoclast development. All three affected siblings were homozygous for a 3 bp inframe deletion in exon 3 of the TNFRSF11B gene, resulting in the loss of an aspartate residue. Their parents (who were first cousins) were heterozygous for the mutation. Recombinant wild-type and mutant OPG cDNAs were expressed in human epithelial kidney cells, and secreted OPG was collected from the conditioned medium. In vitro measurements of bone resorption showed that wild-type OPG suppressed bone resorption, whereas the mutant form did not, confirming this to be an inactivating mutation. This description of abnormal OPG function in humans expands the spectrum of genetic bone diseases arising from perturbations of the OPG/RANK-L/RANK system that regulates osteoclastogenesis.

INTRODUCTION

Idiopathic hyperphosphatasia (MIM 239000, also known as Juvenile Paget’s disease, amongst other synonyms) was first described in 1956 (1). More than 50 cases have been described, and although the genetic basis is unknown, familial occurrence with an autosomal recessive pattern of inheritance is well recognized (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/). On clinical and radiological grounds, the disorder is classified as a craniotubular dysostosis with hyperphosphatasia. It is a generalized skeletal disorder characterized by markedly increased bone turnover. This is manifested histologically by increased numbers of both osteoclasts and osteoblasts in bone, and reflected biochemically by an increased rate of excretion of type I collagen breakdown products, and very high plasma alkaline phosphatase activity, from which the disorder takes its name. The disease is usually recognized in infancy or early childhood, depending on the severity of the phenotype. The long bones develop gross abnormalities, with widened diaphyses and progressive deformities. The axial skeleton is also affected, with vertebral and pelvic deformity. Skull involvement causes massive thickening of the calvarium. Reducing bone turnover with calcitonin or bisphosphonates, which inhibit osteoclastic bone resorption, can permit more...
normal bone to develop, suggesting that the high rates of bone resorption and formation are responsible for the structural abnormalities of the bone (2).

Bone turnover is tightly regulated by many systemic hormones and local factors. One group of molecules, OPG/RANK-L/RANK, acts as a convergence point for many regulatory pathways. The bone-forming osteoblasts express and secrete the tumour necrosis factor-like molecule, RANK-L (receptor activator of nuclear factor \( \alpha \)B ligand) in response to various hormonal, cytokine and mechanical stimuli. RANK-L binds to its receptor RANK (receptor activator of nuclear factor \( \alpha \)B), expressed on the surface of osteoclast precursors. The binding of RANK-L to RANK initiates the differentiation of these precursors to mature, active osteoclasts. Osteoblasts also secrete a decoy receptor, osteoprotegerin (OPG), which acts as an inhibitor of bone resorption by binding to RANK-L, preventing activation of osteoclast precursors through RANK. Animal experiments indicate that imbalances in this system, either a relative excess of RANK-L or OPG, can cause osteoporosis or osteopetrosis, respectively (3).

We describe here a family that presented to Auckland Hospital with three members affected with severe symptoms of idiopathic hyperphosphatasia. Given the large size of the family and the number of affected siblings, we investigated the genetic basis of the increased bone turnover and skeletal deformities in this family.

RESULTS

Clinical presentation

We studied nine members from two generations of a New Zealand family of Iraqi origin. The parents were first cousins and three of their nine children displayed typical clinical, biochemical and radiographic features of idiopathic hyperphosphatasia. (Fig. 1 and Table 1). The unaffected family members were phenotypically normal, apart from short stature (<3rd percentile). In the affected children, difficulty walking had first become evident around the age of 5 years, and by the age of 15 the two older affected siblings were wheelchair-bound. Long-bone fractures occurred in two of the three siblings, but the major skeletal complication was progressive deformity with severe kyphoscoliosis due to vertebral collapse, and acetabular protrusion. Progressive sensorineural deafness was noted from about 8 years. A transiliac bone biopsy from the youngest child had a very unusual anatomical appearance, with almost the entire specimen being made up of parallel trabecular plates (Fig. 2). Quantitative histomorphometry indicated that the proportion of trabecular surfaces undergoing active osteoclastic bone resorption was 2.8%, which is more than three times the median value for this age group (0.9%, range 0.4–1.8%) (4).

Identification of the candidate gene

A genome screen of the family members was undertaken using the ABI PRISM Linkage Mapping Set HD5 (Applied Biosystems). LOD scores of 2.31 and 2.21 were obtained for chromosomes 7q36 and 8q24, respectively. High-density mapping using markers D7S594, D7S559 and D7S2423 for chromosome 7, and 20 markers from the Généthon and Marshfield Linkage databases between and including D8S555 and D8S1799 for chromosome 8, narrowed the interval further (Fig. 3). The HGMD and the NCBI databases were screened at these loci for known genes and expressed sequence tags (ESTs). The TNFRSF11B gene encoding for OPG was selected for further analysis, since it lay within the region 8q24. This gene is strongly expressed in bone, and OPG secreted by osteoblasts plays a key role in regulating osteoclast differentiation (2,5,6).

Identification of a mutation in the TNFRSF11B gene

PCR primers were designed from the intronic sequences flanking the five exons of the OPG gene to enable identification of mutations both in the coding regions and at the splice sites. Sequencing identified a 3 bp inframe deletion in exon 3, predicted to result in the loss of an aspartate residue at position 182 (Fig. 4A). All three affected siblings were homozygous for this mutation. Both parents and two unaffected siblings were
are still growing.

Markers of bone turnover in family members

<table>
<thead>
<tr>
<th>Family member and status</th>
<th>Age (years) at study and sex</th>
<th>Plasma alkaline phosphatase (U/l)</th>
<th>Urine N-telopeptide/creatinine (nmol BCE/mmol creat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent, unaffected</td>
<td>58 M</td>
<td>100</td>
<td>54</td>
</tr>
<tr>
<td>Parent, affected</td>
<td>51 F</td>
<td>99</td>
<td>44</td>
</tr>
<tr>
<td>Sibling, unaffected</td>
<td>28 F</td>
<td>1286</td>
<td>510</td>
</tr>
<tr>
<td>Sibling, affected</td>
<td>25 M</td>
<td>91</td>
<td>41</td>
</tr>
<tr>
<td>Sibling, affected</td>
<td>23 M</td>
<td>1290</td>
<td>2086</td>
</tr>
<tr>
<td>Sibling, unaffected</td>
<td>19 F</td>
<td>52</td>
<td>—</td>
</tr>
<tr>
<td>Sibling, affected</td>
<td>16 F</td>
<td>97</td>
<td>70</td>
</tr>
<tr>
<td>Sibling, affected</td>
<td>11 F</td>
<td>1968</td>
<td>3350</td>
</tr>
<tr>
<td>Sibling, unaffected</td>
<td>12 F</td>
<td>177</td>
<td>206</td>
</tr>
</tbody>
</table>

The siblings are listed in birth order (excluding two older brothers who did not take part in the study). Normal adult ranges: plasma alkaline phosphatase activity, 30–120U/l; urine N-telopeptide/creatinine, 3–51 nmol bovine collagen equivalent (BCE)/mmol creatinine. Normal values are higher in children who are still growing.

heterozygous, and two unaffected siblings were homozygous for the wild-type allele. The mutation was not detected in 100 chromosomes from 50 control subjects. The missing aspartate residue in the mutant, OPGΔD182, is located in the fourth cysteine-rich domain of OPG (Fig. 4B). This aspartate residue is very highly conserved in other members of the tumour necrosis factor receptor (TNFR) superfamily, suggesting that it is required for normal structure and/or function (Fig. 4C) (7,8). Experimental evidence supporting the significance of aspartate 182 has been shown in truncation and deletion analyses, which indicate that this region is crucial for OPG function (see Discussion) (5,9).

Analysis of the function of the mutant protein

OPG was detectable in the plasma of all three affected subjects (1.8–4.7 ng/l, normal range 2–5 ng/l), suggesting that the mutant protein was secreted. In order to determine if the OPG in these patients was biologically active, wild-type human OPG cDNA was cloned into the expression vector pcDNA3.1(−)/myc–HisA and the pOPGΔD182 construct was prepared by site-directed mutagenesis. These constructs were transiently transfected into HEK293 cells. Western blot analysis of conditioned media samples demonstrated bands for both wild-type OPG and the OPGΔD182, but, unexpectedly, the mutant protein had a lower electrophoretic mobility than the wild type (Fig. 5A). When both protein preparations were subjected to deglycosylation with N-glycosidase F, they appeared to be of similar size (Fig. 5B). This suggested that the difference in electrophoretic mobility observed before was due to altered glycosylation of the mutant protein. The biological activities of the wild-type and the mutant proteins were compared in a bone resorption assay in an organ culture system. Hemicalvariae of neonatal mice were incubated in the presence of samples of the conditioned media containing equal concentrations of either OPG or OPGΔD182. The release of 45Ca into the medium is a measure of the resorption activity of the osteoclasts in the calvariae. As shown in Fig. 6, conditioned medium containing wild-type OPG inhibited bone resorption, whereas that containing the OPGΔD182 protein did not.

DISCUSSION

The OPG/RANK-L/RANK system is critical in the regulation of bone turnover. RANK is expressed on osteoclast precursors. Interaction with its ligand RANK-L stimulates cellular differentiation to active, mature osteoclasts. OPG, a soluble decoy receptor secreted by osteoblasts, binds RANK-L, preventing stimulation of osteoclastogenesis (2). We hypothesize that the mutant OPG produced by the affected family members fails to block the interaction between RANK-L and RANK. This is the first human disorder to be described resulting from a mutation in OPG, and it confirms the importance of OPG to bone physiology in humans. The link between a mutation in OPG and idiopathic hyperphosphatasia expands the recognized spectrum of genetic bone disease arising from dysfunction of the RANK/RANK-L/OPG regulatory system. Activating mutations in RANK have been described, and cause the related autosomal dominant conditions of familial expansile osteolysis (MIM 174810) and expansile skeletal hyperphosphatasia (10,11). Patients with these conditions share several features with the family described here, including bone lysis with high bone turnover and hyperphosphatasia. Indeed, the phenotypic similarities are such that one family with familial expansile osteolysis was originally described as having ‘familial Paget’s disease’ (10) and the family with expansile skeletal hyperphosphatasia was originally described as having ‘juvenile Paget’s disease’ (12). Our observations clearly indicate that autosomal recessive idiopathic hyperphosphatasia can arise from inactivating mutations in OPG. It is probable, however, that the clinical diagnosis of idiopathic hyperphosphatasia encompasses a genetically heterogeneous group. A number of other disorders, such as generalized polyostotic fibrous dysplasia, have similar phenotypes but may have different aetiology (13). The mutation described in this work, resulting in the loss of D182, is located within the fourth cysteine-rich domain of OPG. Molecules of the TNFR family include the consensus motif DTV or NTV preceding the last cysteine of each cysteine-rich domain. This motif is critically important in the stabilization of the domain loop structure. These residues are involved in the formation of hydrogen bonds, which anchor the loop structure and stabilize the disulfide bonds (14). The residue deleted in our patients (D182), is the D in this consensus DTV motif, and is conserved in mouse, rat and human OPG. The likely effect of this mutation is an OPG molecule of destabilised structure, failure of the last disulfide bond within this domain to form, and disruption of the loop structure necessary for correct folding of the molecule.

Two groups have investigated the domains of OPG and their importance to function. These studies have shown that only the
The first four domains (encompassing the TNFR-like region) are required for activity in an osteoclastogenesis assay (although with only 10% of the activity of the full-length molecule). The last intrachain disulfide bond within this domain is critical for OPG function (5,9).

The alteration of glycosylation of the mutant protein was unexpected. In other proteins, glycosylation is important for correct folding, trafficking, stabilization and protection from proteases, as well as for interaction with other molecules (15).

The primary sequence of OPG contains five potential glycosylation sites (N-X-T/S): four in the TNFR region and one in the C-terminal region (N98, N152, N165, N178, N289). There is also another rarely used potential glycosylation site at position 183 (N-X-C; N183). Aspartate 182 is in between two potential glycosylation sites. The degree of glycosylation at each of these sites in vivo has not previously been investigated. Altered folding could make the sites flanking the mutation more available for glycosylation than in the correctly folded molecule.

Figure 2. Bone histology (undecalciﬁed specimen) from the youngest affected child. (A) Low-power view of the whole specimen, which consists of parallel trabecular plates with almost no crosslinking, and thin cortices (von Kossa stain, ×45 magnification). (B) For comparison, a section of normal bone illustrating the normal honeycomb pattern of trabecular bone. (C) High-power view of trabecular bone from the youngest affected child. Active osteoclastic bone resorption is present on one side of the trabeculae (large white arrow), whilst on the opposite surface, active osteoblastic bone formation is taking place. The trabecular surface is covered by a thin osteoid layer (small white arrow). On top of the osteoid is a layer of active osteoblasts (small black arrow). Goldner’s trichrome stain, ×450 magnification. Mineralized bone is green and unmineralized osteoid is orange.
OPG knockout mice develop severe, early-onset, high-turnover osteoporosis (16,17). The bones appear normal at birth, but bone loss is evident from trabecular sites by 1 week, and from cortical sites by 4 weeks of age. Histological analysis confirms that the bone loss results from accelerated bone turnover. Older mice develop long bone and vertebral fractures and severe deformity—a natural history similar to that in our patients. Another abnormality in OPG knockout mice is vascular calcification (16). Interestingly, the literature on idiopathic hyperphosphatasia describes patients who developed ‘pseudoxanthoma elasticum’, one feature of which is widespread vascular calcification (18). OPG is now being developed as a potential therapeutic agent for osteoporosis (19), and it would be interesting to observe its effect in our patients, who are effectively OPG-deficient.

**MATERIALS AND METHODS**

**Biochemical analysis**

Estimation of OPG levels used the ELISA kit from Immundiagnostik AG (D-64625 Bensheim, Wiesenstrasse 4, Germany). Total alkaline phosphatase activities in the plasma were measured by autoanalyser. Urinary N-telopeptide (a breakdown product of type I collagen) was measured using...
Figure 4. Identification of a deletion mutation in a highly conserved region of OPG. (A) Alignment of a section of OPG exon 3, showing the sequence electropherograms of a child homozygous for the mutated allele and a heterozygous parent. Only the forward sequences are shown; the reverse sequences were in agreement with these. The arrow indicates the position of the mutation. (B) Cartoon of the genomic organization of the TNFRSF11B genomic contigs, showing splice sites. The arrow indicates the position of the mutation. A cartoon of the structure of the OPG protein is also shown. (C) Alignment of the amino acid sequences of the fourth cysteine-rich domains of members of the TNFR superfamily, indicating the conserved residues: OPG, osteoprotegerin (TNFRSF11B); TNFRII, tumour necrosis factor receptor II; CD40, B-cell-associated molecule CD40 (TNFRSF5); RANK, receptor activator of nuclear factor κB (TNFRSF11A); OX40, OX40 antigen (TNFRSF4); 4-1BB, homologue of mouse 4-1BB (TNFRSF9); NGFR, nerve growth factor receptor (TNFRSF16); conserved cysteine residues are indicated in black boxes. The conserved aspartate residue at position 182 of OPG (deleted in the affected family) is shown shaded in yellow.
the Osteomark NTx assay (Ostex International Inc., Seattle, WA). N-telopeptide excretion is expressed as the ratio of bone collagen equivalent (BCE) (in nmol) to creatinine (in mmol).

**Genotyping**

Peripheral blood samples were collected from family members after obtaining informed consent. We isolated leucocyte DNA using the Puregene DNA/RNA extraction kit (Gentra Systems Inc.). Haplotypes were constructed assuming parsimonious linkage phase. Linkage analysis was performed on the assumption of autosomal recessive inheritance and a disease frequency of 1/10000. Pairwise LOD scores were calculated with the MLINK program of the LINKAGE package (v5.1) (20). DNA samples were amplified individually for each marker using fluorescently labelled primers and were electrophoresed using standard semiautomated methods for microsatellite analysis (ABI Prism 3100, Perkin-Elmer).

**Mutation screening and sequence analysis**

Two genomic clones containing the sequences for TNFRSF11B are contained in the National Center for Biotechnology Information database, GenBank accession nos AB008821 (exon 1) and AB008822 (exons 2–5). The PCR primers were designed from the intronic regions flanking the five exons to enable identification of mutations both in the coding regions and splice sites (≥20 bp between the primer and the exon). The exons were amplified using the following primers (5′–3′):

- **ex1F**, GCAGGCGATACTTCCTGTTGC;
- **ex1R**, TAACTTGAAAGCGGTTTCCTGC;
- **ex2F**, TTCATGCTAAGATGATGCCAC;
- **ex2R**, TCCAGACACATAGTACCTACC;
- **ex3F**, AACGATTTGAGGAGAAGGTAC;
- **ex3R**, TCAACTCAGAGAGAGAGATG;
- **ex4F**, TAAGACCAGCCAACAGAAGGT;
- **ex4R**, CATACATGCAGTCTTGTTCCTGG;
- **ex5F**, GGTGTCACTTAACTCCCTCTC;
- **ex5R**, GATAACGATCCAGATCTGACAG.

PCR product lengths for exons 1–5 are 311, 561, 300, 471 and 759 bp, respectively.

Genomic DNA (20 ng) was amplified in 25 μl of 1× PCR buffer, 0.2 mM dNTPs, 0.2 μM of each oligonucleotide primer and 1 μl of 5 U/μl Taq polymerase. PCR conditions were as follows: initial denaturation at 95°C for 2 min; 94°C for 45 s, 55°C for 30 s, 72°C for 30 s for 35 cycles, followed by a final elongation step at 72°C for 10 min. Sequencing was performed on an automatic sequencer (ABI Prism 377, Perkin-Elmer) according to the manufacturer’s instruction. Sequences were assembled using the DNAStar sequence alignment software. We screened a panel of genomic DNA from 50 control subjects (100 chromosomes) by PCR analysis for mutations in TNFRSF11B using the same primers and conditions as described above.
Construction of plasmids

An expression construct of wild-type OPG was prepared by cloning OPG cDNA, produced by RT–PCR of human osteoblast RNA, into pcDNA3.1(−)/myc–HisA plasmid (Invitrogen). RT–PCR primers were designed from the cDNA sequence contained in the National Center for Biotechnology Information database; GenBank accession no. NM_002546. The primers used to amplify the OPG cDNA were 5’-CTGAGAATTCGGAGACCAC-3’ for the forward primer and 5’-CTGATTGACCTGGGATCTATC-3’ for the reverse primer. The size of the product was 1207 bp, corresponding to bases 74–1280. These primers introduced EcoRI and BamHI restriction sites that were used for cloning the product into pcDNA3.1(−)/myc–HisA. For construction of the deletion mutant plasmid, two primers were designed: NdeF, 5’-CAAGTGTATCATATGCCAAATGC-3’; NdeR, 5’-GTTTCCGGAACATATGTTGTGTTG-3’. NdeF corresponds to the region of the pcDNA3.1(−)/myc–HisA plasmid (from bp 474), while NdeR spans a region in OPG cDNA that includes the 3 bp deletion (marked by an asterisk in the primer sequence) and an NdeI restriction site. All constructs were sequenced on both strands to confirm the introduction of the mutation and to exclude any PCR artefacts.

Production and analysis of recombinant OPG and OPGD182

Human epithelial kidney (HEK293) cells were cultured in OPTI-MEM I-Reduced Serum Medium (GIBCO Invitrogen) for 24 h prior to transfection with FuGENE 6 reagent (Roche) and the appropriate construct. After a further 72 h incubation, the conditioned media were collected, cleared of cellular debris and stored at −80°C. The concentrations of OPG and OPGD182 in the conditioned media were determined by ELISA, using a monoclonal mouse anti-OPG capture antibody and polyclonal goat anti-OPG detection antibodies (R&D Systems). For western blot analysis, the proteins were visualized using anti-His antibodies [Anti-His(C-term)-HRP antibody, Invitrogen]. For the glycosylation studies, samples were treated with a N-glycosidase F deglycosylation kit (Roche), and analyzed on a western blot as described above.

Bioassay measurement of 45Ca release in organ cultures

Prior to use in the bioassays, the conditioned media samples were concentrated 10-fold using Centricon YM-10 (Millipore) molecular weight exclusion columns, with a molecular weight cutoff at 10 kDa. The samples were then filtered to ensure sterility, and the concentrations of the wild-type and mutant proteins were adjusted by dilution in OPTI-MEM I-Reduced Serum Medium. The bone resorption bioassay was performed as previously described (21). Hemicalvariae of neonatal mice were incubated in the presence of samples of the conditioned media, containing equal concentrations of either OPG or OPGD182. A recombinant OPG sample donated by AMGEN Inc. was used as a control for OPG function.

GenBank accession numbers

Human gene for OCIF (OPG) exon1, AB008821; human gene for OCIF (OPG) exons 2–5, AB008822; Homo sapiens TNFRSF11B (osteoprotegerin) mRNA, NM_002546.

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