Lack of Circulating Pigment Epithelium-Derived Factor Is a Marker of Osteogenesis Imperfecta Type VI

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Background: Osteogenesis imperfecta (OI) type VI is a rare autosomal recessive bone fragility disorder that is caused by inactivating mutations in SERPINF1, the gene that encodes pigment-epithelium derived factor (PEDF). Determining PEDF serum levels might facilitate the diagnosis of OI type VI.

Objective: The objective of the study was to assess whether lack of circulating PEDF is a specific marker of OI type VI and to evaluate whether PEDF serum levels are influenced by other metabolic bone diseases.

Materials and Methods: Serum PEDF concentrations were measured in 12 patients with OI type VI (aged 2.7–31 yr) as well as in 96 children and adolescents with OI types I, III, and IV; in 26 young patients with hypophosphatemic rickets; and in 19 healthy controls.

Results: Circulating PEDF was undetectable in all 12 patients with OI type VI but was measurable for the other 141 study participants. No significant differences in serum PEDF concentrations were found between the diagnostic groups other than OI type VI. Treatment with bisphosphonates (in OI types I, III, and IV) and with phosphate and calcitriol (in hypophosphatemic rickets) did not have a detectable influence on serum PEDF. In patients with OI types I, III, and IV, serum creatinine, body mass index z-score, and OI severity were significant predictors of PEDF serum levels.

Conclusion: Determining PEDF serum concentration helps to diagnose OI type VI but does not seem to provide information on the activity of bone turnover or mineralization. (J Clin Endocrinol Metab 97: E1550–1556, 2012)
defect in bone tissue that leads to the accumulation of unmineralized osteoid, as seen in osteomalacia (6). These characteristic features of OI type VI are readily apparent on bone histology, but there are no distinguishing radiological signs of OI type VI, and parameters of calcium and phosphorus metabolism are within normal limits. In contrast to other OI types with severe bone fragility, patients with OI type VI usually do not have fractures or bone deformity at birth but typically sustain their first fracture after the age of 6 months and have an increasing fracture rate thereafter. Compared with other types of OI, bisphosphonate treatment appears to have less effect on fracture rate in OI type VI (7).

Recent research has demonstrated that OI type VI is caused by loss-of-function mutations in SERPINF1 (8–11). SERPINF1 is located on chromosome 17p13.3 and codes for pigment-epithelium derived factor (PEDF), a secreted 50-kDa protein that is almost ubiquitously expressed (12). Even though PEDF is a member of the serpin (serine protease inhibitors) family of proteins because of its structural similarity to other members of this group, it does not act as a protease inhibitor (13). PEDF was first discovered in retinoblastoma cell lines and is one of the most potent antiangiogenic factors in humans (14, 15). Adipocytes also produce large amounts of PEDF and serum concentrations of PEDF are elevated in obesity (16–18). In addition, PEDF is expressed in bone cells, particularly at sites of active bone growth, but its function in bone is not well characterized (19, 20).

In a previous study, we had noted that PEDF was absent from the circulation in three patients with OI type VI (9), but it is unknown whether this applies to OI type VI in general. It is also unknown whether the absence of PEDF is specific for OI type VI or can also occur in other types of OI or in other disorders that lead to disturbed bone mineralization. In the present study, we therefore determined PEDF serum concentrations in all OI type VI patients who had been included in the original description of this disorder (6) as well as in several newly identified patients. We also quantified serum concentrations of PEDF in patients with OI types I, III, and IV, in patients with hypophosphatemic rickets and in healthy control subjects.

Subjects and Methods

Subjects

The study population comprised 153 individuals (82 males, 71 females; age range 1.6–31 yr) who were assessed at the Shriners Hospital for Children (Montréal, Québec, Canada). This included 12 patients with a diagnosis of OI type VI, 96 patients who were diagnosed with OI types other than OI type VI, 26 patients with hypophosphatemic rickets, and 19 healthy subjects.

The OI type VI group (age range 2.7–31 yr) included all eight patients who were presented in the original description of OI type VI (6) as well as four newly diagnosed patients (Table 1). Patients 1–11 had undergone iliac bone biopsies, and the diagnosis of OI type VI was made on the basis of typical bone histology findings, as described (a mineralization defect in the presence of normal parameters of systemic mineral metabolism, fish-scale lamellae) (6). In patient 12, no bone biopsy was obtained, but the diagnosis was suspected on the basis of clinical features (healthy appearance at birth, first fracture after the age of 6 months, white sclera, normal looking teeth) and confirmed by SERPINF1 sequence analysis. All but patient 12 had received iv bisphosphonate treatment (pamidronate or zoledronic acid) before the determination of PEDF serum levels.

The group of OI patients suffering from OI types other than OI type VI was subdivided into patients who had previously received iv bisphosphonate treatment with pamidronate or zoledronic acid and patients who had not received bisphosphonate

### TABLE 1. Mutations in the SERPINF1 gene found in 12 patients with OI type VI

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Origin</th>
<th>Mutation</th>
<th>Diagnosis basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>French-Canadian</td>
<td>c.[295C&gt;T]+[295C&gt;T] p.(Arg99*)+(Arg99*)</td>
<td>Bone histology</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>French-Canadian</td>
<td>c.[295C&gt;T]+[295C&gt;T] p.(Arg99*)+(Arg99*)</td>
<td>Bone histology</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>French-Canadian/Irish</td>
<td>c.[271_279dup]+[295C&gt;T] p.(Ala91_Ser93dup)+(Arg99*)</td>
<td>Bone histology</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>Nicaraguan</td>
<td>c.[271–279dup]+[271–279dup] p.(Ala91_Ser93dup)</td>
<td>Bone histology</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>French-Canadian/German</td>
<td>c.[295C&gt;T]+[295C&gt;T] p.(Arg99*)+(Arg99*)</td>
<td>Bone histology</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>Inuit from north Québec</td>
<td>No mutation found</td>
<td>Bone histology</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>German/Polish</td>
<td>c.[271_279dup]+[-Za] p.(Ala91_Ser93dup)+[-Za]</td>
<td>Bone histology</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>French-Canadian (relative of patients 1 and 2)</td>
<td>c.[295C&gt;T]+[295C&gt;T] p.(Arg99*)+(Arg99*)</td>
<td>Bone histology</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>French-Canadian</td>
<td>c.[295C&gt;T]+[295C&gt;T] p.(Arg99*)+(Arg99*)</td>
<td>Bone histology</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>Nepalese</td>
<td>c.[829_831del]+[829_831del] p.(Phe277del)+[Phe277del]</td>
<td>Clinical presentation/SERPINF1 sequencing</td>
</tr>
</tbody>
</table>

Patients 1–8 in this table correspond to patients 1–8 in the report by Glorieux et al. (6). M, Male; F, female.
treatment. The bisphosphonate-treated group was comprised of 67 patients (age range 1.4–22 yr) who had the following clinical diagnoses: OI type I, n = 27; OI type III, n = 13; OI type IV, n = 27. Results of DNA sequence analysis of COL1A1 and COL1A2 were available in 60 patients and revealed disease-causing mutations in 45 patients. Among the 15 patients with negative COL1A1 and COL1A2 sequence analyses, two were positive for homozygous mutations in LEPRE1, leading to recessive OI (3). In the 13 patients with negative sequencing results and in the seven patients without DNA sequence analysis results, the diagnosis of OI was clinically asserted by the association of frequent fractures, low bone mass, and blue or gray sclera or dentinogenesis imperfecta. The group of OI patients who had not been exposed to bisphosphonates included 29 individuals (age range 3–18 yr) who were all diagnosed with OI type I. Twenty of these patients had a known pathogenic mutation in COL1A1 or COL1A2. In four patients, sequence analysis of all exons of these two genes was negative and in five patients, genetic testing had not been performed.

The hypophosphatemic rickets group included 26 patients (age range 1.6–19 yr). The diagnosis of hypophosphatemic rickets was based on the presence of rickets in the clinical and radiological evaluation and low serum phosphorus levels, but normal serum concentrations of calcium, PTH, and 25-hydroxyvitamin D. Sequence analysis of the PHEX gene had revealed disease-causing mutations in 14 patients. In nine patients, sequence analysis of all the exons of the PHEX gene did not show abnormalities, and in three patients, analysis of the PHEX gene had not been performed. At the time of the present study, 15 patients were receiving treatment with phosphate supplementation and calcitriol and 11 had discontinued this therapy because they had reached their final height.

The control group included 19 individuals (age range 6–22 yr) who had been assessed at the Shriners Hospital because they had a family member with a metabolic or genetic bone disorder that was caused by a known mutation. In the individuals who were included in the control group, the presence of the mutation found in their family member had been ruled out. The study was approved by the Institutional Review Board of McGill University and informed consent was obtained from the participants or, in minors, their legal guardians. Assent was obtained from children and adolescents aged 7–17 yr.

**Anthropometric measurements**

Height was measured using a Harpenden stadiometer (Hol- tain, Crymch, UK). Weight was determined using digital electronic scales for infants and mechanical scales for older children and adults (Healthometer, Bridgeview, IL). Height, weight, and body mass index (BMI) were converted to age- and sex-specific z-scores on the basis of reference data published by the Centers for Disease Control and Prevention (21).

**Sequence analysis of SERPINF1**

Total genomic DNA was isolated from peripheral blood or saliva. All eight exons of the SERPINF1 gene, including the exon-intron boundaries, were amplified by PCR. The sequencing reaction was performed using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The nucleotide sequence was determined using an Applied Biosystems 3100 DNA sequencer. Patient sequences were referenced to the Ensembl gene sequence ENSG00000132386 (SERPINF1).

In the genomic DNA of two brothers (patients 8 and 9), no PCR product could be obtained from exon 5. Sequencing of SERPINF1 cDNA (obtained from skin fibroblasts of patient 9) revealed that the sequence corresponding to exon 5 was missing. When PCR was performed on genomic DNA using primers located in exon 4 and intron 5 of SERPINF1, a PCR product was obtained that was approximately 1300 nucleotides shorter than expected from the SERPINF1 consensus sequence. The sequencing of this PCR product demonstrated a 1,310-bp deletion (position on chromosome 17: 1,674,583 to 1,675,892). The deleted region encompassed the entire exon 5.

Mutations for which there was no prior entry in the database of OI mutations (http://www.le.ac.uk/ge/collagen/) were regarded as novel (1, 2).

**Biochemical measurements**

PEDF was measured with an ELISA (BioVendor Laboratory Medicine Inc., Brno, Czech Republic) (22). The intraassay and interassay coefficients of variation were 3.6 and 5.9%, respectively. Serum total calcium, phosphate, alkaline phosphatase, creatinine, and liver enzymes were measured using standard laboratory methods. Serum active intact PTH (fragment 1–84) was determined by RIA (Diasorin, Stillwater, MN). The 25-hydroxyvitamin D125I RIA kit (Diasorin) was used to determine 25-hydroxyvitamin D serum concentrations. Urine creatinine concentration was determined colorimetrically. Urinary cross-linked N-telopeptides of type I collagen, a marker of bone resorption, were quantified by ELISA (Osteomark; Ostex International Inc., Seattle, WA) using the second void sample of the morning. Results were related to urinary creatinine and expressed as a percentage of age- and sex-specific mean reference values using published reference data (uNTX/Cr%) (23). All samples were obtained between 0800 and 1000 h after an overnight fast.

**Dual-energy x-ray absorptiometry**

Lumbar spine areal bone mineral density was determined in the anterior-posterior direction at the lumbar spine (L1-L4) using a Hologic QDR 4500 device (Hologic Inc., Waltham, MA). The results were transformed to age-specific z-scores combining reference data from Salle et al. (24), Southard et al. (25), and Glastre et al. (26).

**Statistical analyses**

Raw results were transformed to age- and sex-specific z-scores from the average result in the reference population, using the published reference data cited in the description of each measurement technique. Differences between two groups were tested for significance using the unpaired Student’s t test. ANOVA was used to compare more than two groups. Group differences in dichotomous variables were tested for significance using the $\chi^2$ test. Stepwise multiple regression analysis was used to assess potential predictors of serum PEDF levels. Nominal variables were coded as follows: gender, male = 1, female = 2; OI severity, OI type I = 1, OI type IV = 2, OI type III = 3. All tests were two tailed and throughout the study, $P$ values <0.05 were considered significant. These calculations were performed using the PASW Statistics software version 18.0 (SPSS Inc., Chicago, IL).
found, but no second sequence abnormality in SERPINF1 was detected. In patient 6, no sequence abnormality was found upon sequencing of the coding region of SERPINF1 including exon/intron boundaries.

In contrast to patients with OI type VI, PEDF serum levels were in the measurable range for each of the other 141 study participants. Serum PEDF levels ranged from 2.7 to 25.3 mg/liter in the 96 patients who had clinical diagnoses of OI types I, III, or IV. The 26 patients with hypophosphatemic rickets had PEDF serum concentrations between 1.6 and 23.6 mg/liter. Healthy controls had serum PEDF levels between 2.0 and 17.5 mg/liter.

To assess whether PEDF levels are influenced by metabolic bone disorders other than OI type VI, we determined PEDF serum concentrations in OI type I, III, and IV patients with and without a history of bisphosphonate treatment as well as in patients with hypophosphatemic rickets and in healthy controls (Table 2). No significant difference in mean PEDF serum concentrations was found between these groups (P = 0.30), even though they differed significantly with regard to z-scores of height, weight, and lumbar spine areal bone mineral density as well as in serum concentrations of phosphorus, creatinine, alkaline phosphatase, PTH, and uNTX/Cr% (Table 2 and Fig. 1B). The differences in PEDF serum levels between these groups remained nonsignificant after adjustment for sex and age (P = 0.18). Among patients with hypophosphatemic rickets, PEDF levels were similar in the patients who were currently receiving treatment with phosphate and calcitriol and patients who had discontinued this treatment.

Multiple regression analysis was performed within the group of 67 OI type I, III, and IV patients receiving bisphosphonates to assess which of a set of putative determinants (age, sex, OI severity, BMI z-score, lumbar spine areal bone mineral density z-score, uNTX/Cr%, serum

### Results

Each of the 12 patients with a diagnosis of OI type VI had undetectable PEDF serum concentrations (<0.3 mg/liter; Fig. 1). In 10 of these patients, homozygous or compound heterozygous mutations in SERPINF1 were detected (Table 1). The mutations found in patients 8, 9, and 12 were novel. In one patient (patient 7), a heterozygous mutation was identified in the coding region of SERPINF1 including exon/intron boundaries.

#### TABLE 2. Clinical characteristics of the study groups

<table>
<thead>
<tr>
<th></th>
<th>OI type VI</th>
<th>OI + BP</th>
<th>OI – BP</th>
<th>HPR</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>12 8/4</td>
<td>12 6/6</td>
<td>26 13/13</td>
<td>19 9/10</td>
<td>0.01</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>16.4 (8.4)</td>
<td>12 6.4 (4.4)</td>
<td>26 13.2 (5.5)</td>
<td>19 12.7 (4.9)</td>
<td>0.50</td>
</tr>
<tr>
<td>Height (z-score)</td>
<td>12 -4.9 (3.6)</td>
<td>26 -0.8 (1.1)</td>
<td>19 -0.5 (0.7)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Weight (z-score)</td>
<td>12 -1.4 (1.9)</td>
<td>26 -0.6 (1.1)</td>
<td>19 -0.1 (0.9)</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>BMI (z-score)</td>
<td>12 1.0 (1.5)</td>
<td>26 -0.1 (1.4)</td>
<td>19 0.1 (1.1)</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>LS-aBMD (z-score)</td>
<td>12 -2.6 (1.6)</td>
<td>26 1.1 (1.6)</td>
<td>19 0.1 (1.1)</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>Calcium (mmol/liter)</td>
<td>12 2.39 (0.11)</td>
<td>26 2.38 (0.10)</td>
<td>19 2.42 (0.07)</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Phosphorus (mmol/liter)</td>
<td>12 1.37 (0.16)</td>
<td>26 1.54 (0.26)</td>
<td>19 1.51 (0.26)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Creatinine (umol/liter)</td>
<td>12 27 (10)</td>
<td>26 47 (20)</td>
<td>19 51 (19)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase (U/liter)</td>
<td>12 203 (96)</td>
<td>26 225 (112)</td>
<td>19 204 (98)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>PTH (pmol/liter)</td>
<td>12 2.1 (1.5)</td>
<td>26 1.9 (0.7)</td>
<td>19 4.7 (4.0)</td>
<td>1.72 (1.8)</td>
<td></td>
</tr>
<tr>
<td>25-Hydroxyvitamin D (nmol/liter)</td>
<td>11 61 (22)</td>
<td>25 72 (17)</td>
<td>9 78 (14)</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>uNTX/Cr%</td>
<td>10 102 (62)</td>
<td>26 86 (44)</td>
<td>9 125 (44)</td>
<td>9 114 (33)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The P value indicates the significance (by ANOVA) of the difference between the following groups: OI + bisphosphonate treated, OI no bisphosphonates, hypophosphatemic rickets, and controls. OI +BP, OI patients treated with bisphosphonates; OI –OI, OI patients not exposed to bisphosphonates; HPR, hypophosphatemic rickets; NA, not available; LS-aBMD, lumbar spine areal bone mineral density; M, male; F, female.
PEDF serum levels, sequence analysis of VI. In a patient with severe bone fragility and undetectable serum concentrations is a useful screening test for OI type all had detectable PEDF serum levels.

In this study, we found that all participants with a diagnosis of OI type VI had PEDF serum levels below the detection limit. This was true for all eight patients who had been included in our initial description of this disorder (6) as well as for the patients who we diagnosed with OI type VI thereafter. Our results also show that the absence of circulating PEDF is specific for OI type VI because 96 patients with other types of OI, 26 patients with hypophosphatemic rickets, and 19 healthy control subjects had detectable PEDF serum levels.

These results indicate that the determination of PEDF serum concentrations is a useful screening test for OI type VI. In a patient with severe bone fragility and undetectable PEDF serum levels, sequence analysis of SERPINF1 is thus likely to find a disease-causing mutation. Our observations suggest that the absence of circulating PEDF may be a more sensitive diagnostic indicator of OI type VI than sequence analysis of all exons and exon/intron boundaries of SERPINF1. Indeed, two of our patients had undetectable PEDF in serum, but DNA sequence analysis did not resolve the cause of the disease because in one patient only a heterozygous mutation was detected, and in another patient no sequence abnormality was found at all. It is possible that these patients have disease-causing SERPINF1 mutations that escaped detection with the methods used, such as intronic mutations or heterozygous exon deletions.

Even though serum PEDF seems to be a sensitive diagnostic marker of OI type VI, our study does not suggest that measuring circulating PEDF concentrations provides information about the activity of bone metabolism or about the mineralization process. Patients with OI types I, III, and IV and patients with hypophosphatemic rickets had similar PEDF serum levels as healthy controls. Bisphosphonate treatment, which decreases bone remodeling activity in OI (27), and treatment of hypophosphatemic rickets with phosphate and calcitriol did not seem to have an effect on PEDF serum levels. Our results in all groups were similar to those that were observed in other studies on healthy subjects that used the same assay to quantify PEDF (22). No significant association was found between serum PEDF and the urinary excretion of N-telopeptide of collagen type I, a marker of bone resorption.

The origin of the mineralization defect in OI type VI is still unclear; the known functional properties of PEDF do not provide obvious explanations why the lack of PEDF should result in a delayed mineralization (12, 28). There is no evidence that abnormalities in vitamin D play a role because 25-hydroxyvitamin D levels in OI type VI patients were similar to the other patient groups and serum levels of calcium, phosphorus, and PTH are within normal limits in OI type VI (6). We have previously found no evidence that 25-hydroxyvitamin D levels in the range from 13 to 103 nmol/liter are associated with measures of bone mineralization in children and adolescents with OI (29).

Even though no obvious relationship between serum PEDF and bone metabolism could be detected, our analyses in patients with OI types I, III, and IV showed that serum PEDF was positively associated with serum creatinine and BMI z-score and negatively with OI severity. The associations of PEDF serum levels with serum creatinine and BMI z-score were expected. Studies in healthy adults have consistently shown correlations between serum concentrations of PEDF and creatinine (30, 31). The mechanistic origin of this correlation has not been investigated, but both creatinine and PEDF are produced and released by skeletal muscle cells (32, 33). Other studies have shown that PEDF is secreted by adipocytes and that PEDF serum levels are higher in obese individuals, which explains the association between serum PEDF and BMI z-scores (16–18).

The negative association between PEDF levels and OI severity is more difficult to account for. One possible explanation is that OI types vary with regard to PEDF expression or metabolism. However, it is also possible that low PEDF levels in the most severely affected patients simply reflects lower fat mass and that fat mass in this patient group was not captured by BMI z-score in the same manner as on the other groups. Severely affected OI patients often have short or deformed legs, which may cause BMI results to be falsely elevated because leg deformity leads to lower body height, and BMI is inversely related to the square of the body height.

The present results suggest that lack of circulating PEDF is a common characteristic of OI type VI, regardless of the nature of the underlying SERPINF1 mutation. It might therefore become possible to devise a
treatment of OI type VI that is based on PEDF replacement treatment. However, before such a treatment can be devised, the differential roles of circulating PEDF and locally produced PEDF on bone metabolism need to be determined. The present study, in any case, does not point toward a major influence of circulating PEDF on bone metabolism.

Conclusion
Determination of PEDF serum levels helps to diagnose OI type VI but does not seem to provide information on the activity of bone turnover or mineralization.

Acknowledgments
We thank Patty Mason for the determination of PEDF serum activity of bone turnover or mineralization. Determination of PEDF serum levels helps to diagnose OI treatment. However, before such a treatment can be devised, the differential roles of circulating PEDF and locally produced PEDF on bone metabolism need to be determined. The present study, in any case, does not point toward a major influence of circulating PEDF on bone metabolism.

Conclusion
Determination of PEDF serum levels helps to diagnose OI type VI but does not seem to provide information on the activity of bone turnover or mineralization.

References

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