The effect of SERPINF1 in-frame mutations in osteogenesis imperfecta type VI⁎

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A B S T R A C T
Osteogenesis imperfecta type VI is caused by mutations in SERPINF1, which codes for pigment-epithelium derived factor (PEDF). Most of the reported SERPINF1 mutations lead to premature termination codons, but three in-frame insertion or deletion mutations have also been reported. It is not clear how such in-frame mutations lead to OI type VI. In the present study we therefore investigated how SERPINF1 in-frame mutations affect the intracellular localization and secretion of PEDF. Skin fibroblasts affected by SERPINF1 in-frame mutations transcribed SERPINF1 at slightly reduced levels but secretion of PEDF was markedly diminished. Two deletions (p.F277del and the deletion of SERPINF1 exon 5) were associated with retention of PEDF in the endoplasmic reticulum and a stress response in osteoblastic cells. A recurrent in-frame duplication of three amino acids (p.Ala91_Ser93dup) appeared to lead to intracellular degradation but no retention in the endoplasmic reticulum or stress response. Immunofluorescence imaging in transiently transfected osteoblastic MC3T3-E1 cells suggested that PEDF affected by in-frame mutations was not transported along the secretory pathway. MC3T3-E1 osteoblasts stably overexpressing SERPINF1 with the p.Ala91_Ser93dup mutation had decreased collagen type I deposition and mineralization. Thus, the assessed homozygous in-frame deletions or insertions lead to retention or degradation within cellular compartments and thereby interfere with PEDF secretion.

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Introduction

Osteogenesis imperfecta (OI) is a heritable bone fragility disorder that in the large majority of cases is caused by dominant mutations in COL1A1 or COL1A2, the genes coding for collagen type I alpha chains [1]. The resulting phenotype is traditionally classified into four OI types (I to IV), largely depending on the severity of bone fragility. Additional OI types (V and higher) have more recently been identified, initially based on specific phenotypic characteristics and then on the basis of genetic findings.

One of these ‘newer’ OI types – OI type VI – is caused by recessive loss-of-function mutations in SERPINF1 (MIM 172860) [2–5], the gene coding for pigment-epithelium derived factor (PEDF) [6]. PEDF is a 418 amino acid secreted glycoprotein that is thought to be involved in many biological processes, such as the regulation of angiogenesis, adipogenesis and bone formation, and which binds to extracellular matrix proteins such as collagen and glycosaminoglycans. OI type VI had initially been identified on the basis of a specific bone histological picture that included an increased amount of unmineralized osteoid, suggesting that PEDF is also involved in mineralization of bone matrix [7]. Nevertheless, it is not clear how SERPINF1 mutations lead to bone fragility.

About 30 individuals with OI type VI have been reported until now, carrying 18 unique SERPINF1 mutations (http://www.le.ac.uk/ge/collagen/) [2–5,8–15]. While most of the reported SERPINF1 mutations are frameshift (n = 8) or nonsense (n = 5) mutations that are expected to lead to mRNA instability due to nonsense-mediated decay, three in-frame insertion or deletion mutations have also been reported (Fig. 1). These are a recurrent 9-nucleotide duplication leading to the addition of three amino acids (p.Ala91_Ser93dup; 91_93dup) [5,10], a recurrent 3-nucleotide deletion causing the elimination of one amino acid (p.F277del; ΔF277) [10,14], and a deletion of exon 5 (ΔE5) that leads
to a deletion of 66 amino acid residues coupled with the insertion of an arginine residue (p.Lys147_Gly215delinsArg) [10]. The 91_93dup and ΔF277 mutations affect highly conserved residues and the deletion of exon 5 eliminates a positively charged region of the protein (Fig. 1).

These in-frame mutations are particularly interesting from a mechanistic point of view, as it is expected that mRNA carrying an in-frame mutation is stable and that protein is produced. It is therefore not clear how in-frame mutations in SERPINF1 lead to OI type VI. In the present study we therefore investigated how SERPINF1 in-frame mutations affect the intracellular localization and secretion of PEDF.

Subjects and methods

Subjects

Study participants were patients with a diagnosis of OI type VI who were assessed at the Shriners Hospital for Children in Montreal. The study was approved by the Institutional Review Board of McGill University and informed consent was obtained from the participants or, in the case of minors, their legal guardians. Assent was obtained from children and adolescents aged 7 to 17 years. Clinical data were obtained by retrospective chart review.

Clinical data

Height was measured using a Harpenden stadiometer (Holtain, Crymych, UK). Height and weight were converted to age- and sex-specific z-scores on the basis of reference data published by the Centers for Disease Control and Prevention [16].

Serum PEDF was quantified with an enzyme-linked immunosorbent assay (BioVendor Laboratory Medicine Inc., Brno, Czech Republic). The intra-assay and inter-assay coefficients of variation were 3.6% and 5.9%, respectively. The assay was also used to determine PEDF concentration in conditioned medium of skin fibroblast cell cultures.

For sequence analysis of SERPINF1, total genomic DNA was isolated from peripheral blood or saliva. Sequence analyses for SERPINF1 mutations were performed either by Sanger sequencing using an Applied Biosystems 3100 DNA sequencer after PCR amplification of all exons and exon-intron boundaries, or by semiconductor-based next-generation sequencing using an Ion Torrent PGM device (Life Technologies), as described previously [17]. Patient sequences were compared to NCBI Reference Sequence NM_002615.5. The mutations examined in the present study have been previously reported [10].

Generation of mutant PEDF

To generate PEDF with the 91_93dup, ΔF277, and ΔE5 mutations, cDNAs encoding the wild type and mutant forms of PEDF were cloned by reverse-transcribed PCR using skin fibroblast RNA from affected individuals. The cDNAs were used for PCR amplification using a primer set (forward 5′-atccagcgccgagctcgg-3′; reverse 5′-catattaggggcccctggggt-3′) that covered nucleotides 142–1418 of the SERPINF1 reference sequence (NM_002615). PCR products were cloned into the CMV-promoter driven backbone of pcDNA as described [18]. All constructs were confirmed by Sanger sequencing. For transient transfection experiments, cells were transfected with plasmid DNA (1 μg/well) using XtremeGENE 9 (Roche). Cells were collected 48 h thereafter, or fixed for immunofluorescence microscopy as described below.

Cell culture

Skin fibroblast cultures were maintained and grown in Dulbecco’s modified Eagle’s minimal essential medium supplemented with 50 μg/ml ascorbic acid (Life Technologies). MC3T3-E1 and HEK293 cells were transiently transfected with equal amounts of expression plasmids. For the stable overexpression of mutant PEDF in MC3T3-E1 osteoblasts, SERPINF1 coding sequence carrying the 91_93dup mutation was introduced into lentiviral expression vector (pLVX-puro), which was then used for lentivirus production by transient transfection in HEK293 cells as described [19]. Conditioned medium containing the virus was collected and concentrated by ultracentrifugation. The concentrated viruses were used to transduce MC3T3-E1 cells. After 24 h, puromycin selection was started at a concentration of 5 μg/ml and continued for 4 days. The selected transduced cell line was then expanded to start the osteoblast functional studies. Control MC3T3-E1 cells were transduced with a lentivirus generated with the empty pLVX-puro vector.

Collagen was quantified using picrosirius red staining. Soluble calf skin collagen type I (Sigma) was used for generating standard curves. Mineral deposition was visualized by von Kossa staining. For tissue non-specific alkaline phosphatase staining, cells were fixed with 100% methanol and then stained with a ready-to-use alkaline phosphatase substrate solution (Sigma).
**RNA extraction and real-time qPCR analyses**

Real-time PCR assessment of gene expression was performed on an ABI 7500 Real-Time PCR Machine using cDNA transcripts prepared from 2 μg RNA, and the TaqMan Universal PCR Master Mix and Taqman primers specific for SERPINF1 (Hs01106934_m1) and β-actin (4352935E) (all from Life Technologies). Transcript levels were normalized to β-actin levels using the threshold cycle method and values were expressed as $2^{-\Delta \Delta Ct}$. These values were then used to convert ΔCt values to mRNA fold differences relative to controls.

**Protein preparations and Western blotting**

Proteins from cell lysates or from conditioned medium were separated on 10% SDS-polyacrylamide gels. After transfer onto PVDF membranes, primary antibodies were applied against PEDF (Millipore), GRP78 (Abcam), and β-actin (Thermo Scientific). The membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody. The separated protein bands were visualized by using Amersham ECL prime Western blotting detection reagent (GE Healthcare Life Sciences).

**Immunofluorescence microscopy**

Cell layers were fixed with 3.7% formaldehyde. Cultures were blocked with 2% bovine serum albumin (BSA, Sigma), and then stained using antibodies against PEDF or GRP78. This was followed by incubation with Alexa Fluor-tagged secondary antibodies against rabbit, or mouse, antibodies with wavelength excitations of 488 nm (green) or 594 nm (red) (Invitrogen). Nuclei were visualized with DAPI (Invitrogen). Cells were mounted with Gold anti-Fade medium (Invitrogen), dried overnight, and observed with a Leica DM 4000B upright fluorescence microscope.

**Results**

**Clinical characteristics**

Homozygous SERPINF1 in-frame mutations were observed in 4 patients (P1 to P4; Table 1). All of these individuals were born without fracture or bone deformity, but 3 patients sustained the first fracture in their first year of life. All patients had a history of a large number of long-bone fractures and were short to a variable degree. Serum levels of PEDF were at or below the level of detection in all 4 patients.

**Analyses in skin fibroblasts (Fig. 2)**

Skin fibroblasts from patients P1 to P4 were compared to those of a healthy control (‘positive control’) and three patients with OI type VI who were homozygous for stop mutations in SERPINF1 (‘negative controls’). In P1 to P4, SERPINF1 transcript levels were slightly lower than in control fibroblasts, whereas SERPINF1 transcript was undetectable in fibroblasts carrying the homozygous nonsense mutation. Western blot and ELISA showed that PEDF was secreted into the media by control fibroblasts, but PEDF was barely detectable in the media of fibroblast cultures harboring SERPINF1 mutations, regardless of mutation type. As expected for a secreted protein, control fibroblasts did not have detectable amounts of PEDF in the cell layer under the conditions tested here. The same was true for 91_93dup or ΔE5 fibroblasts. Given

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**Table 1**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Amino acid change</th>
<th>First Fx (y)</th>
<th>Last FU (y)</th>
<th>Height (z-Score)</th>
<th>Weight (z-Score)</th>
<th>Fx (N)a</th>
<th>Fx/year (N)b</th>
<th>PEDF (mg/l)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 M</td>
<td>p.Ala91_Ser93dup</td>
<td>0.9</td>
<td>16.1</td>
<td>−4.9</td>
<td>0.0</td>
<td>38</td>
<td>2.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>P2 M</td>
<td>p.Lys147_Gly215delinsArg</td>
<td>0.8</td>
<td>18.4</td>
<td>−9.4</td>
<td>−4.1</td>
<td>13</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>P3 M</td>
<td>p.Lys147_Gly215delinsArg</td>
<td>3.0</td>
<td>12.3</td>
<td>−1.8</td>
<td>0.3</td>
<td>9</td>
<td>1.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>P4 M</td>
<td>p.Phe277del</td>
<td>0.9</td>
<td>3.0</td>
<td>−4.4</td>
<td>−4.1</td>
<td>6</td>
<td>2.9</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Abbreviations: Fx, fracture; FU, follow-up; PEDF, pigment-epithelium derived factor.

a Lifetime number of fractures (femur, tibia and humerus only).

b Lifetime number of fractures (femur, tibia and humerus only) divided by the age at last follow-up visit.

c Healthy controls (n = 19): 2.0 to 17.5 mg/l. Data for height, weight, fractures and PEDF were obtained at the time of the last follow-up visit.
the presence of SERPINF1 mRNA transcripts, the absence of detectable protein in either cell layer or conditioned medium suggests that mRNA affected by 91_93dup or ΔE5 is either poorly translated or that the protein is degraded rapidly. In contrast, ΔF277 fibroblasts had detectable PEDF within the cells.

**Transient PEDF overexpression experiments (Fig. 3)**

As the main clinical characteristic of OI type VI is defective bone formation, we assessed the fate of PEDF in osteoblasts by overexpressing wild type and mutant PEDF in MC3T3-E1 cells, an osteoblastic cell...
line. In cells transfected with wild type PEDF, immunofluorescence microscopy showed staining of both endoplasmic reticulum (ER) and Golgi elements, consistent with PEDF following a secretory pathway (Fig. 3A). Labeling for GRP78 – an ER-resident protein also known as BiP or HSPA5 – gave a characteristic reticular and evenly distributed ER signal. Cells expressing 91_93dup showed punctate vesicular staining with very little or no clear ER or Golgi labeling. This pattern was even more pronounced when ΔE5 was overexpressed, where labeling for both PEDF and GRP78 overlapped perfectly, without defined typical ER/Golgi structures. In contrast, ΔF277 was restricted to a pure ER-labeling pattern, overlapping with GRP78.

The effect of PEDF mutations on PEDF secretion was assessed in HEK293 cells, because the high-level endogenous PEDF secretion of MC3T3-E1 osteoblasts made results difficult to interpret in these cells. Compared to cells transfected with wild type PEDF, PEDF secretion was markedly decreased by 91_93dup and ΔE5, and was undetectable after transfection with ΔF277 (Fig. 3B). In contrast, mutated PEDF was present in the cell layer. The ER stress marker GRP78 was upregulated in cells expressing PEDF carrying ΔE5 or ΔF277 (Fig. 3C).

Stable PEDF overexpression in MC3T3-E1 osteoblasts (Fig. 4)

91_93dup is a recurrent mutation that has been observed in individuals from a variety of ethnic backgrounds [5,10]. We therefore selected 91_93dup for a more detailed characterization through stable overexpression in MC3T3-E1 osteoblasts (Fig. 4A). Compared to cells transfected with empty vector, MC3T3-E1 cells overexpressing 91_93dup deposited 30% less collagen (Fig. 4B), produced fewer mineralization nodules and less alkaline phosphatase activity (Fig. 4C). In contrast, overexpression with wild type PEDF did not have a clear effect on collagen deposition, but led to increased production of mineralization nodules and higher alkaline phosphatase staining (Fig. 4C).

Discussion

The present study shows that skin fibroblasts affected by SERPINF1 in-frame mutations translated SERPINF1 at slightly reduced levels but secreted no or very little PEDF. Our experiments in osteoblastic MC3T3-E1 cells suggested that PEDF affected by in-frame mutations was not transported along the secretory pathway, explaining the lack of secretion.

Even though skin fibroblasts harboring SERPINF1 in-frame mutations secreted no or very little PEDF, HEK293 cells could be forced to secrete mutated PEDF in transient overexpression experiments. However, these cells secrete mutated PEDF at much lower levels than cells overexpressing wild type PEDF, despite the fact that similar amounts of mutated and wild type PEDF were present in the cell layer. This indicates that mutated PEDF is translated, but that the mutation affected secretion of PEDF. Immunofluorescence microscopy of transiently overexpressing MC3T3-E1 cells showed that all three mutated PEDF forms fail to be transported along the secretory pathway, explaining the lack of secretion. Subtle differences in the pattern of staining were noted, however, among the different forms of mutated PEDF. The 91_93dup and ΔE5 mutations led to punctate vesicular staining, while ΔF277 was mostly retained in the ER.

The ΔE5 and ΔF277 mutations were associated with increased levels of GRP78, which implies that cells expressing these mutant forms of PEDF suffer from ER stress. Cellular retention and up-regulated expression of chaperones, such as GRP78, has been demonstrated in several studies on mutated proteins causing skeletal dysplasias [20–23]. It is therefore possible that ER stress contributes to the pathogenicity of the ΔE5 and ΔF277 mutations.

Stable overexpression of the recurrent 91_93dup mutation in MC3T3-E1 cells clearly had a negative effect on these cells, as it interfered with collagen deposition and mineralization. Previous biochemical analyses in cultured fibroblasts with SERPINF1 nonsense and frameshift mutations have not found evidence for abnormalities in collagen type I [2]. It is possible that the effect of SERPINF1 mutations differs between osteoblasts and skin fibroblasts, given that individuals who are homozygous for such mutations have a severe bone disorder but apparently normal skin. Also, stable overexpression forced the MC3T3-E1 cells to express high levels of mutated protein, which may have induced detrimental effects that are not detectable when the endogenous SERPINF1 is mutated. Nevertheless, MC3T3-E1 cells overexpressing wild type PEDF had normal collagen secretion, suggesting that the lower collagen deposition in cells overexpressing the 91_93dup mutation is specific for mutated PEDF.

Apart from decreased collagen deposition, MC3T3-E1 cells overexpressing the 91_93dup mutation have decreased alkaline phosphatase activity and a defect in matrix mineralization, whereas overexpressing wild type PEDF had the opposite effects. Together, these results suggest that overexpressing mutated PEDF interferes with cellular differentiation or function, whereas wild type PEDF accelerates differentiation or function in these osteoblastic cells.

In conclusion, the assessed homozygous SERPINF1 in-frame deletions or insertions lead to retention or degradation within cellular compartments and thereby interfere with PEDF secretion.

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**Fig. 4.** Stable overexpression of wild type (WT) and 91_93dup PEDF in MC3T3-E1 osteoblast cultures. Controls cells (C) were transfected with empty vector. A. Similar levels of human SERPINF1 mRNA expression are observed in cells transfected with WT (results set to 1.0) and 91_93dup SERPINF1. No human SERPINF1 mRNA is detected in control cells (not shown). The Western blot shows that cells transfected with WT SERPINF1 secrete more PEDF into the medium than control cells. However, cells overexpressing 91_93dup PEDF secrete less PEDF than control cells. B. Collagen deposition is 30% less in 91_93dup PEDF-overexpressing cells. Error bars represent the standard deviation of triplicate measurements. C. Van Kossa staining (upper panels) and staining for tissue nonspecific alkaline phosphatase (TNAP, lower panels) show that overexpressing 91_93dup PEDF resulted in less mineralized extracellular matrix and less alkaline phosphatase activity as compared to control cell cultures, whereas overexpression of wild type PEDF results in more mineralization nodules and more alkaline phosphatase activity.
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Roles of the authors: HA performed in vitro studies and devised the first draft of the manuscript; TP and FHG contributed patient information; PR supervised sequence analyses; MDM revised the manuscript content; PM supervised in vitro studies and revised the manuscript content; FR conceptualized the project, contributed patient information, finalized the report and accepts responsibility for the integrity of the data analysis. All authors have read and approved of the final version of the manuscript.

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