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Restoration of the serum level of *SERPINF1* does not correct the bone phenotype in *Serpinf1* null mice

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ABSTRACT

Osteogenesis imperfecta (OI) is a group of genetic disorders characterized by bone fragility and deformity. OI type VI is unique owing to the mineralization defects observed in patient biopsies. Furthermore, it has been reported to respond less well to standard therapy with bisphosphonates [1]. Others and we have previously identified *SERPINF1* mutations in patients with OI type VI. *SERPINF1* encodes pigment epithelium derived factor (PEDF), a secreted collagen-binding glycoprotein that is absent in the sera of patients with OI type VI. *Serpinf1* null mice show increased osteoid and decreased bone mass, and thus recapitulate the OI type VI phenotype. We tested whether restoration of circulating PEDF in the blood could correct the phenotype of OI type VI in the context of protein replacement. To do so, we utilized a helper-dependent adenoviral vector (HDAd) to express human *SERPINF1* in the mouse liver and assessed whether PEDF secreted from the liver was able to rescue the bone phenotype observed in *Serpinf1*$^{-/-}$ mice. We confirmed that expression of *SERPINF1* in the liver restored the serum level of PEDF. We also demonstrated that PEDF secreted from the liver was biologically active by showing the expected metabolic effects of increased adiposity and impaired glucose tolerance in *Serpinf1*$^{-/-}$ mice. Interestingly, overexpression of PEDF in vitro increased mineralization with a concomitant increase in the expression of bone gamma-carboxyglutamate protein, alkaline phosphatase and collagen, type I, alpha I, but the increased serum PEDF level did not improve the bone phenotype of *Serpinf1*$^{-/-}$ mice. These results suggest that PEDF may function in a context-dependent and paracrine fashion in bone homeostasis.

1. Introduction

Osteogenesis imperfecta (OI) is a group of heterogeneous genetic disorders largely characterized by bone fragility and deformity. Among the different types of OI, the recessively inherited OI type VI is unique owing to the mineralization defects observed in patient biopsies. Individuals with OI type VI display a dramatic increase in osteoid and prolonged mineralization lag time, and show variable response to standard treatment with bisphosphonates [1,2]. Whole exome studies have identified loss of function mutations in *SERPINF1* as causative of OI type VI [3–5]. *SERPINF1* encodes pigment epithelium derived factor (PEDF), a 50 kDa collagen-binding glycoprotein that belongs to the serine protease inhibitor superfamily [2]. PEDF was first isolated from the retina and was described as having potent neurotropic and antiangiogenic activity [6]; recent reports support a potential anti-tumor role for the protein in many forms of cancers [7,8].

Serpinf1$^{-/-}$ mice recapitulate the hypomineralization and low bone mass phenotypes observed in patients with OI type VI, and are thus an excellent model for therapeutic studies [9]. In this study, we examined whether restoration of PEDF into the serum using a helper dependent adenoviral (HDAd) system was able to correct the skeletal defects observed in the *Serpinf1*$^{-/-}$ mice. Helper-dependent adenoviral vectors are devoid of viral genes, and hence, exhibit dramatically lower host adaptive immune response. In multiple animal models including nonhuman primates, a single injection can lead to life-long sustained expression from the liver [10]. *SERPINF1* expression was mediated using the HDAd approach owing to the ability of the vector system to transduce hepatocytes and secrete PEDF into the serum [11].

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2. Materials and methods

2.1. Ethics statement

All research involving animals was approved by the Center for Comparative Medicine, in conjunction with the Institutional Animal Care and Use Committee, and conducted according to the relevant national and international guidelines. Veterinarians supervised animal care according to the standards of the Baylor College of Medicine Center for Comparative Medicine, a program fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Animals

The *Serpinf1* −/− mice were a generous gift from S. Crawford and were generated as described previously [12]. The *Serpinf1*$^{-/-}$ mouse colony was maintained in a C57Black/6J genetic background and housed in the Baylor College of Medicine Animal Vivarium. All of the mice were housed under pathogen-free conditions; food and water were provided ad libitum. Mice used in the experiments were males and between 7 and 9 weeks of age. All experimental procedures were conducted in accordance with institutional guidelines for animal care and use.

2.3. Cell culture, virus production and stable cell generation

HEK293T and MC3T3-E1 cells were cultured in DMEM or α-MEM medium with 10% FBS, 1% glutamine and 1% penicillin–streptomycin. The *SERPINF1* cDNA was cloned into the expression plasmid pLenti CMV Puro DEST (w118-1), a gift from Eric Campeau (Addgene plasmid #17452). 293T cells were used to generate a lentivirus constitutively expressing *SERPINF1*, as previously described [13]. Briefly, pLenti*SERPINF1*PuroDEST was cotransfected with the psPAX2 and pMD2.G packaging vectors using Superfect (Qiagen). The supernatant was collected at 36 and 48 h post transfection. After centrifugation, viral particles were concentrated overnight at 4 °C using PEG5000 and resuspended in 10 mM Tris after centrifugation at 4 °C. MC3T3-E1 cells were transduced with the virus; after 48 h, cells were selected with medium containing 5 μg/ml puromycin for a period of 2 weeks to obtain clones that stably expressed *SERPINF1*.

2.4. Western blotting and mineralization assay

Alizarin red staining assays were performed as previously described on MC3T3 cells stably expressing *SERPINF1* [13]. A total of 15 μl of conditioned media was electrophoresed and transferred onto PVDF membranes (Millipore). The membranes were blocked with 5% milk in Tris buffered saline/0.1% Tween (TBST) and incubated with a 1:1000 dilution of primary antibodies for PEDF (Millipore). Primary antibodies were detected with appropriate HRP-conjugated secondary antibodies, and the signal was detected using a chemiluminescence detection kit (Pierce).

2.5. RNA extraction and real time RT-PCR

Total RNA from MC3T3 cells overexpressing *SERPINF1* was extracted using TRIzol (Life Technologies). Samples were treated with DNase (Roche), and the Superscript III First Strand RT-PCR kit (Life Technologies) was used to synthesize cDNA. qRT-PCR was performed on a LightCycler instrument (Roche), with *Gapdh* as the internal control to normalize gene expression. Three independent replicates and one technical replicate were included for each analysis. Two-tailed, unpaired t-tests with unequal variances were used for statistical analysis.

2.5.1. Viral vector construction, production and injection in animals

HDAd HDΔ25.3E4PEPCK-*SERPINF1* constructs containing the human *SERPINF1* cDNA transgene driven by the liver-restricted phosphoenolpyruvate carboxykinase (PEPCK) promoter (HDAd-*SERPINF1*) were produced as described elsewhere [14,15]. Purified HDAds were diluted in phosphate buffered saline (PBS) and injected into tail veins, with a total volume of 200 μl. All injections were done in the mornings. Blood was collected retro-orbitally, and plasma and serum samples were frozen immediately and stored at −80 °C until analysis. Once the animals were sacrificed, the livers were harvested and kept at −80 °C until analysis.

2.6. ELISA

Concentrations of serum PEDF were determined per manufacturer's protocol using the commercial human PEDF ELISA kit (Biovendor).

2.7. Intraperitoneal glucose tolerance test

Mice were fasted overnight, with access to plenty of water. After measuring the baseline glucose level, 10% glucose solution per gram bodyweight was injected intraperitoneally. Glucose levels were measured using a glucose meter at 15, 30, 60 and 120 min post injection.

2.8. Animal tissue collection, processing and histology

Serpinf1$^{-/-}$ mice and wild-type littermates were sacrificed at 6 months of age. Liver, femurs and spines were dissected, fixed, embedded, and sectioned according to methods previously described [13]. Routine histologic analysis of the tissues was performed according to standard protocols. ANOVA was used to analyze fat pad weights (Graphpad Prism).

2.9. Micro-computed tomography (μCT)

For μCT, spine and femur samples were placed into a 16 mm tube filled with 70% ethanol and scanned, at a resolution of 16 μm, using a ScanCo μCT40 scanner (N = 4–5, males). Trabecular and cortical bone analysis was performed using the ScanCo software, as previously described [13]. Two-tailed, unpaired t-tests with unequal variances were used for statistical analysis.

3. Results

3.1. Overexpression of SERPINF1 in MC3T3 cells results in enhanced osteoblast differentiation and mineralization

Since loss of *Serpinf1* in mice results in a mineralization defect, we first assessed whether overexpression of *SERPINF1* would have an effect on mineralization. MC3T3 cells were transduced with a lentivirus overexpressing *SERPINF1*. Real-time PCR data showed increased expression of human *SERPINF1*, but not of mouse *Serpinf1* (Fig. 1a). Western blot analysis using conditioned medium from MC3T3 cells overexpressing *SERPINF1* shows a band of approximately 50 kDa, which suggests that cells secreted PEDF (Fig. 1b). In comparison to control MC3T3 cells, cells overexpressing *SERPINF1* exhibited a high degree of mineralization as shown by alizarin red staining (Fig. 1c). Real-time PCR analysis revealed a concomitant increase in the expression of osteoblast differentiation markers, bone gamma-carboxyglutamate protein (*Bglap*), alkaline phosphatase (*Alp*), and collagen, type I, alpha I (*Col1a1*) (Fig. 1d). Together, these results suggest that the overexpression of *SERPINF1* increases mineralization and is bioactive in this in vitro context.