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Preservation of Type H Vessels and Osteoblasts by Enhanced Preosteoclast Platelet-Derived Growth Factor type BB Attenuates Glucocorticoid-Induced Osteoporosis in Growing Mice

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Abstract

Survival of chronic diseases in childhood is often achieved utilizing glucocorticoids, but comes with significant side effects, including glucocorticoid-induced osteoporosis (GIO). Knowledge of the mechanism of GIO is limited to the adult skeleton. We explored the effect of genetic loss and inhibition of cathepsin K (*Ctsk*) as a potential treatment target in a young GIO mouse model as genetic loss of cathepsin K results in a mild form of osteopetrosis secondary to impaired osteoclast bone resorption with maintenance of bone formation. We first characterized the temporal osteoclast and osteoblast progenitor populations in *Ctsk*^{-/-} and wild type (WT) mice in the primary and secondary spongiosa, as sites representative of trabecular bone modeling and

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Disclosure

All authors state that they have no conflicts of interest.

remodeling, respectively. In the primary spongiosa, *Ctsk*^{-/-} mice had decreased numbers of osteoclasts at young ages (2 and 4 weeks) and increased osteoblast lineage cells at later age (8 weeks) relative to WT littermates. In the secondary spongiosa, *Ctsk*^{-/-} mice had greater numbers of osteoclasts and preosteoblasts relative to WT littermates. We next developed a young GIO mouse model with prednisolone 10 mg/m²/day injected intraperitoneally daily from 2 through 6 weeks of age. Overall, WT-prednisolone mice had lower bone volume per tissue volume, whereas *Ctsk*^{-/-}-prednisolone mice maintained a similar bone volume relative to *Ctsk*^{-/-}-vehicle controls. WT-prednisolone mice exhibited a decreased number of osteoclasts, tartrate-resistant acid phosphatase and platelet-derived growth factor type BB (PDGF-BB) co-positive cells, type H endothelial cells, and osteoblasts relative to WT-vehicle mice in both the primary and secondary spongiosa. Interestingly, *Ctsk*^{-/-}-prednisolone mice demonstrated a paradoxical response with increased numbers of all parameters in primary spongiosa and no change in secondary spongiosa. Finally, treatment with a cathepsin K inhibitor prevented WT-prednisolone decline in osteoclasts, osteoblasts, type H vessels, and bone volume. These data demonstrate that cells in the primary and secondary spongiosa respond differently to glucocorticoids and genetic manipulation. Inhibition of osteoclast resorption that preserves osteoclast coupling factors, such as through inhibition of cathepsin K, may be a potential preventive treatment strategy against GIO in the growing skeleton.

Keywords

Glucocorticoid induced osteoporosis; bone vasculature; mice; postnatal growth

Introduction

Skeletal mass increases rapidly during childhood, doubling during adolescence [1, 2]. In the metaphysis of the growing skeleton, the cartilage template of the growth plate is invaded by blood vessels, osteoclasts, and osteoblasts to form the primary spongiosa [3–6]. The primary spongiosa is modeled by osteoclast bone resorption and osteoblast bone formation at independent sites to build trabecular bone to maintain the strength of the ends of long bone, termed secondary spongiosa. In bone remodeling, which occurs predominantly in the secondary spongiosa, bone formation and resorption are coupled to maintain trabecular bone throughout adulthood [7]. Osteoclast precursors secrete and release factors from the bone matrix during resorption that alter the bone marrow microenvironment to recruit endothelial precursor cells and mesenchymal stem cells for angiogenesis and osteogenesis, respectively, to support osteoblast bone formation [8–10]. Factors that regulate the cells of the primary spongiosa are just beginning to be elucidated [11].

Survival of many chronic diseases in childhood is achieved utilizing glucocorticoids. However, the survival comes at a cost to the growing skeleton, resulting in impairment of the acquisition of peak bone mass and is the major etiology of secondary osteoporosis in children. Glucocorticoid induced osteoporosis (GIO) has an incidence of 6–10% and prevalence of 29–45% in children taking chronic high dose glucocorticoids [12]. The impact of glucocorticoids on bone modeling has not been well described [5], whereas the effect on bone remodeling has been extensively studied. Prior studies on the effects of glucocorticoids have limited trabecular bone analyses to areas of bone remodeling to specifically eliminate

the potential confounding effects of glucocorticoids on linear and radial growth [13]. In the secondary spongiosa, glucocorticoids at supraphysiological doses have detrimental effects on the skeleton due to direct effects on both osteoclast and osteoblast lineages. Glucocorticoids increase survival of osteoclasts in the short term, although differentiation is suppressed with chronic use [14–16]. Glucocorticoids also induce apoptosis and reduce proliferation and differentiation of osteoblasts and induce osteocyte apoptosis [13, 17, 18]. Glucocorticoids also affect the bone microenvironment, such as detrimental effects on vasculature [18, 19]. As the process of bone mass accrual in childhood involves both modeling and remodeling, both need to be understood more fully when considering pharmaceutical targets to treat childhood GIO.

Signals in the bone marrow microenvironment can be manipulated to increase or decrease both bone modeling and remodeling [9, 10, 20]. In pycnodysostosis, a rare, mild form of osteopetrosis due to loss of function mutations in cathepsin K (*Ctsk*), osteoclasts are unable to resorb the cartilage bone matrix, but an increased number of osteoclasts are observed [21, 22]. We have previously shown that preosteoclasts secrete platelet-derived growth factor type BB (PDGF-BB), which recruits endothelial and mesenchymal progenitor cells to couple angiogenesis and osteogenesis, respectively, at both bone modeling and remodeling sites [10]. PDGF-BB is increased in global *Ctsk* knockout mice (*Ctsk*^{-/-}) [10]. In the present study, we first characterized osteoclasts and osteoblast precursors in the primary and secondary spongiosa regions of wild type (WT) littermates relative to *Ctsk*^{-/-} mice during postnatal growth. We then established a young mouse model of GIO to evaluate the effect of glucocorticoids in *Ctsk*^{-/-} and WT littermates in both the primary and secondary spongiosa. Overall, *Ctsk*^{-/-} mice were resistant to GIO. Osteoclasts, osteoblasts, and blood vessels demonstrated a unique phenotype between primary and secondary spongiosa in response to deletion of *Ctsk* and chronic high daily prednisolone. WT mice treated with prednisolone had a decreased number of tartrate-resistant acid phosphatase positive (TRAP⁺), TRAP⁺/PDGF-BB⁺, type H, and osteocalcin⁺ cells relative to vehicle treated mice in both the primary and secondary spongiosa. However, *Ctsk*^{-/-}-prednisolone mice demonstrated a paradoxical response with increased numbers of all parameters in the primary spongiosa and no change observed in the secondary spongiosa relative to vehicle treated *Ctsk*^{-/-} mice. Finally, the GIO phenotype in WT-prednisolone mice was prevented using the cathepsin K inhibitor. In vitro studies showed that higher concentrations of PDGF-BB enhance VEGF in endothelial cells and restores endothelial tube formation and can overcome effects of prednisolone. These data suggest that inhibition of osteoclast resorption that preserves osteoclast coupling factors, such as PDGF-BB, may be a potential preventive treatment strategy against GIO in the growing skeleton.

Methods

Mouse generation

We generated *Ctsk*^{-/-} and WT littermates by crossing two heterozygote strains as previously described (outbred 129SVJ-C57BL/6J background) [22]. Wild type mice (C57BL6–B6/129X1 background) previously purchased from Jackson Laboratory (Bar Harbor, ME) were used for the cathepsin K inhibitor experiments. We determined the genotype of the

mice by PCR analyses of genomic DNA isolated from mouse tails using the following primers: *Ctsk* forward, 5'-GCCACACCCACACCCTAGAAG-3' and reverse, 5'-ACAAGTGTACATTCCCGTACC-3'. For the characterization of osteoclast and osteoblast lineage cells of the primary and secondary spongiosa, *Ctsk*^{-/-} mice and WT littermates were sacrificed at 2, 4, and 8 weeks. For the GIO studies, two-week-old *Ctsk*^{-/-} mice and WT littermates were randomly divided to receive either vehicle or prednisolone (Sigma, St. Louis, MO) 10 mg/m²/day injected intraperitoneally daily and sacrificed at 6 weeks of age to specifically evaluate the effect of steroids on the growing skeleton. Body surface area (m²) was utilized rather than weight to account for the high metabolic rate in young mice and to allow direct extrapolation for comparison of the dose utilized in mice to humans [23, 24]. Body surface area was calculated using Meeh's formula, using a k constant of 9.82 for mice x body weight (g) to the two-thirds power (BSA=kW^{2/3}) [25]. For the prevention of GIO studies, we treated 2-week-old WT mice daily with both prednisolone (Sigma, St. Louis, MO) 10mg/m²/day and 20 mg kg⁻¹ b.w. L-235 (Merck) for 4 weeks. L-235 was administered intraperitoneally in a 0.5% Methocel (w/v) suspension. All experiments utilized 6 mice per group (3 males, 3 females) unless otherwise specified. Femora and tibia were collected and processed as described for subsequent experiments. We maintained all animals in the Animal Facility of the Johns Hopkins University School of Medicine, housed in gangs in sterile, ventilated cages (Allentown Caging Equipment, Allentown, NJ), allowed free access to water and standard mouse chow, on a 14/10 hour light/dark cycle. No adverse events occurred in the experimental animals. The Animal Facility complies with the National Institutes of Health Animal Welfare Act and maintains appropriate policies and procedures to ensure humane care and use of animals. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Johns Hopkins University, Baltimore, MD, USA.

Microcomputed tomography (μCT) analysis.

Femora were dissected from mice, fixed overnight in 70% ethanol and analyzed by high-resolution μCT (Skyscan 1172, Skyscan). The scanner was set at a voltage of 49 kV, a current of 200 μA and a resolution of 8.7 μm per pixel. We used image reconstruction software (NRecon v1.6), data analysis software (CTAn v1.9) and three-dimensional model visualization software (μCTVol v2.0) in order to analyze the parameters of the distal femoral metaphyseal trabecular bone. We established cross-sectional images of the femora to perform three-dimensional histomorphometric analyses of the trabecular bone. Trabecular bone region of interest was drawn starting from 5% of femoral length proximal to distal epiphyseal growth plate and extended proximally for a total of 5% of femoral length. The trabecular bone was segmented from the bone marrow and analyzed to determine the trabecular bone volume fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N) and trabecular separation (Tb. Sp).

Bone Immunofluorescence and immunohistochemistry

Freshly dissected bone tissues were immediately fixed in ice-cold 4% paraformaldehyde solution for 4 h. For frozen sections, decalcification was carried out with 0.5 M EDTA at 4°C with constant shaking for 24 h. Decalcified bones were immersed into 20% sucrose and 2% polyvinylpyrrolidone (PVP) solution for 24 h. The tissues were then embedded and

frozen in 8% gelatin (porcine) in presence of 20% sucrose and 2% PVP (for 30µm thick section, endomucin & CD31 co-staining) or in optimal cutting temperature (OCT) compound (for 10µm thick section, PDGF-BB & TRAP co-staining). For immunofluorescence staining and morphological analyses, sections were generated using low-profile blades on a Leica CM3050. For paraffin embedded sections, the tissues were fixed in 10% buffered formalin for 48 h, decalcified in 10% EDTA (pH 7.4) (Amresco) for 21 d and embedded in paraffin or OCT compound (Sakura Finetek).

For phenotypic analyses, *Ctsk*^{-/-} mice or WT-L-235 treated mice and their respective controls were always processed, sectioned, stained, imaged and analyzed together at the same conditions and settings. We processed four-micrometer-thick longitudinally oriented paraffin sections of bone including the metaphysis and diaphysis for TRAP staining using a staining kit (Sigma-Aldrich), immunohistochemistry for osteocalcin and osterix staining. We performed immunofluorescence analysis for endomucin & CD31 co-staining and PDGF-BB & TRAP co-staining. For immunostaining, bone sections were air-dried, permeabilized for 10 min in 0.3% Triton X-100, blocked in 5% donkey serum at room temperature for 1 hour. Bone sections were incubated with individual primary antibodies to mouse CD31 (Abcam, ab28364, 1:50), endomucin (Santa Cruz, V.7C7, 1:100), TRAP (Santa Cruz, sc-30833, 1:100), PDGF-BB (Abcam, ab21234, 1:100), osteocalcin (Takara, M173, 1:50), or osterix (Abcam, ab22552, 1:100) overnight at 4 °C. Subsequently, we used secondary antibodies conjugated with fluorescence (1:250) at room temperature for 1 hour while avoiding light. We used isotype-matched controls, such as polyclonal rabbit IgG (R&D Systems, AB-105-C), polyclonal goat IgG (R&D Systems, AB-108-C) and monoclonal rat IgG2A (R&D Systems, 54447) under the same concentrations and conditions as negative controls. Nuclei were counterstained with DAPI for immunofluorescence and hematoxylin for immunohistochemistry. Sections were thoroughly washed with PBS before mounting them using FluoroMount-G (Southern Biotech). Finally, coverslips were sealed with nail polish.

Image acquisition and quantitative analysis

Immunofluorescence co-staining of endomucin & CD31 were analyzed at high resolution with a Zeiss laser scanning confocal microscope, LSM-780 or LSM-510. Z-stacks of images were processed with Imaris software (version 7.00, Bitplane). Leica TCS SP1 or SP2 confocal microscope or an Olympus BX51 Microscope were used for imaging other samples. We counted the number of positively stained cells in three high magnification visual fields in trabecular bone per femur in three sequential sections per mouse in each group of both the primary and secondary spongiosa. The primary spongiosa was defined as the bone adjacent to the growth plate, whereas the distance from primary to secondary spongiosa was defined at 0.5 mm at 2 weeks, and 1 mm for 4 and 8 weeks, similarly to previous reports [26, 27].

ELISA analysis

Bone marrow was collected from bilateral tibia and forelimbs, after surgical removal of growth plate by centrifugation at 4,000 rpm for 10 min at 4° C. Samples were stored at -80° C until analyzed. We performed PDGF-BB ELISA analysis of bone marrow supernatant

using a Mouse/Rat PDGF-BB Quantikine ELISA kit (R&D Systems) according to the manufacturers' instructions.

In vitro assays for tube formation by endothelial precursor cells (EPCs)

We plated Matrigel (BD Biosciences) in 96-well culture plates and incubated at 37 °C to polymerize for 45 min. We then seeded EPCs (2×10^4 cells/well) on polymerized Matrigel plates. Four groups of cells were incubated in medium with additional PBS as a negative control or 10^{-6} M prednisolone (Sigma, St. Louis, MO) or 60 ng/ml PDGF-BB (Pepro Tech, INC) or both prednisolone and PDGF-BB. After incubation at 37 °C for 4 hours, we observed tube formation by microscopy and measured the cumulative tube length. Results were representative of three independent experiments.

Quantitative real-time PCR

Total RNA for quantitative real-time (qRT)-PCR was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA purity was tested by measuring the absorbance at 260 and 280 nm. For qRT-PCR, cDNA was prepared with random primers using the SuperScript FirstStrand Synthesis System (Invitrogen) and analyzed with SYBR GreenMaster Mix (Qiagen, Valencia, CA) in the thermal cycler with two sets of primers specific for each targeted gene. Relative expression was calculated for each gene by 2^{-CT} method, with GAPDH for normalization. Primers used for qRT-PCR are listed in Supplementary Table 1.

Statistical analysis

All error bars are s.d. Data presented as mean $\pm$ s.d. We used paired, two-tailed Student's t-tests for comparisons between two groups and ANOVA for multiple comparisons. All data demonstrated a normal distribution and similar variation between groups. For all experiments, $P < 0.05$ were considered to be significant and indicated by '*'; $P < 0.01$ were indicated by '**'; $P < 0.001$ were indicated by '***'. All inclusion/exclusion criteria were preestablished and no samples or animals were excluded from the analysis. The experiments were randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Results

Osteoclasts and osteoblast lineage cells in primary spongiosa demonstrate unique phenotype during postnatal development relative to secondary spongiosa

To understand the impact of genetic manipulation of cathepsin K on bone modeling during postnatal growth, we quantitated osteoclasts and osteoblast lineage cells in the primary spongiosa of WT and *Ctsk*^{-/-} littermates at 2, 4, and 8 weeks of age. The region of interest for the primary spongiosa was defined as area of tibia trabecula cancellous bone adjacent to the growth plate. The number of osteoclasts, as indicated by TRAP⁺ cells per trabecular area, was significantly decreased in 2 and 4 week old *Ctsk*^{-/-} relative to WT mice at similar ages (Fig. 1A–B). The number of TRAP⁺ cells in the primary spongiosa was increased in *Ctsk*^{-/-} mice at 8 weeks relative to 2 and 4 weeks of age, such that no difference was noted in TRAP⁺ cell number between *Ctsk*^{-/-} and WT mice at 8 weeks (Fig. 1A–B). The number

of osteoblast lineage cells, as indicated by osterix-positive (Osx⁺) staining, in primary spongiosa decreased from 2 to 4 to 8 weeks in WT mice (Fig. 1C–D). While *Ctsk*^{-/-} Osx⁺ cells decreased from 2 to 4 weeks, no change was noted between 4 and 8 weeks of age such that the number of osteoblast lineage cells was significantly increased in *Ctsk*^{-/-} relative to WT littermates at 8 weeks of age (Fig. 1C–D). Overall, in the primary spongiosa, trabecular area per tissue area of tibia did not differ in WT and *Ctsk*^{-/-} mice until 8 weeks of age (Fig. 1E, Supplementary Table 2). Therefore, genetic deletion of *Ctsk*^{-/-} did not significantly alter bone modeling until 8 weeks of age, a late pubertal stage in mice.

To evaluate bone remodeling during postnatal growth, we quantitated osteoclasts and osteoblast lineage cells in the secondary spongiosa of WT and *Ctsk*^{-/-} littermates at 2, 4, and 8 weeks of age. The region of interest for the secondary spongiosa was defined as area of tibia trabecula cancellous bone 0.5 (2 weeks) or 1 mm (4 and 8 weeks) distal from growth plate. The number of TRAP⁺ cells per trabecular area remained similar at 2 and 4 weeks of age, declining at 8 weeks of age in the secondary spongiosa in WT mice, whereas the TRAP⁺ cell number did not significantly decrease in *Ctsk*^{-/-} mice between 2, 4 and 8 weeks (Fig. 2A–B). As a result, the number of TRAP⁺ cells was increased in *Ctsk*^{-/-} relative to WT mice at 8 weeks of age (Fig. 2A–B). The number of osteoblast lineage cells, as indicated by Osx⁺ cells remained similar in WT mice during postnatal growth, whereas fluctuations were observed in the *Ctsk*^{-/-} mice with the highest number of osteoblast lineage cells noted at 2 weeks and lowest number at 4 weeks (Fig. 2C–D). Given the fluctuation of Osx⁺ cells in *Ctsk*^{-/-} mice, the number of osteoblast lineage cells were increased relative to WT littermates at 2 and 8 weeks of age (Fig. 2C–D). Overall, in the secondary spongiosa, trabecular area per tissue area of tibia was increased at all time points in *Ctsk*^{-/-} mice relative to WT littermates (Fig. 2E; Supplementary Table 2). Therefore, genetic deletion of *Ctsk*^{-/-} significantly altered parameters of bone remodeling during postnatal growth.

Genetic loss of *Ctsk* protects young mice from glucocorticoid-induced osteoporosis of the femur

As glucocorticoids have direct effects on both osteoclasts and osteoblasts, promoting bone resorption and impairing bone formation [28], we examined the effect of glucocorticoids in young mice femoral trabecular bone. We first established a young-GIO mouse model by injecting 2 week old WT mice with either vehicle or prednisolone 10 mg/m²/day for 2 or 4 weeks. WT mice exposed to 10 mg/m²/day of prednisolone for 4 weeks had lower femoral trabecular bone volume per tissue volume, decreased trabecular number, and increased trabecular space, but no difference in trabecular thickness in the distal femur metaphysis relative to vehicle treated WT mice (Fig. 3A–E). *Ctsk*^{-/-} mice have increased bone volume secondary to impaired osteoclast bone resorption, but maintenance of osteoblast bone formation [10, 21]. To evaluate if impaired osteoclast function could augment the osteoporotic phenotype, we utilized *Ctsk*^{-/-} mice and injected with either vehicle or prednisolone 10 mg/m²/day from 2 to 6 weeks of age. WT male mice were noted to weigh less than *Ctsk*^{-/-} male littermates between postnatal day 14 and 30, however prednisolone had no effect on weight relative to vehicle-exposed mice (Suppl. Fig. 1A). No differences were noted in female mice weight regardless of genotype or treatment group (Suppl. Fig. 1A). Additionally, no differences were noted in nasoanal length of either males or females

(Suppl. Fig. 1B). *Ctsk*^{-/-} mice treated with vehicle had higher bone volume per tissue volume, increased trabecular number, and decreased trabecular space relative to WT littermates exposed to vehicle (Fig. 3A–E), similar to previous reports [10, 22]. Interestingly, *Ctsk*^{-/-} mice treated with prednisolone had higher bone volume per tissue volume relative to *Ctsk*^{-/-} mice treated with vehicle, but no change in other parameters (Fig. 3A–E), demonstrating resistance to detrimental effects of glucocorticoids on bone volume.

***Ctsk*^{-/-} mice maintained TRAP⁺ cell production of PDGF-BB after exposure to chronic daily high dose prednisolone**

To characterize the effect of glucocorticoids on bone modeling and remodeling, we then systematically characterized femoral trabecular bone cell changes in primary and secondary spongiosa of WT and *Ctsk*^{-/-} littermates injected with prednisolone 10 mg/m²/day from 2 through 6 weeks of age relative to vehicle treated littermates. In vehicle treated mice, the number of TRAP⁺ cells did not differ in the primary spongiosa (Fig. 4A), but was increased in the secondary spongiosa of *Ctsk*^{-/-} mice relative to WT littermates (Fig. 4B). Chronic daily high dose prednisolone led to a significant decrease in TRAP⁺ cells in WT mice relative to WT-vehicle mice in both the primary and secondary spongiosa (Fig. 4A–B). However, *Ctsk*^{-/-} mice exposed to daily high dose prednisolone had increased TRAP⁺ cells in the primary spongiosa (Fig. 4A) and no difference in the secondary spongiosa relative to *Ctsk*^{-/-}-vehicle littermates (Fig. 4B).

We have previously reported that TRAP⁺ cells produce PDGF-BB to couple angiogenesis and osteogenesis with bone resorption during bone modeling and remodeling [10]. At 6 weeks of age, there was no difference between the number of cells co-staining for TRAP and PDGF-BB in WT-vehicle relative to *Ctsk*^{-/-}-vehicle mice in either the primary (Fig. 5A) or secondary spongiosa (Fig. 5B). Prednisolone treatment led to a decreased number of TRAP⁺/PDGF-BB⁺ cells in both the primary and secondary spongiosa in WT mice relative to vehicle treatment (Fig. 5A–B). However, *Ctsk*^{-/-} mice exposed to daily high dose prednisolone had increased number of TRAP⁺/PDGF-BB⁺ cells in the primary spongiosa (Fig. 5A) and no difference in the secondary spongiosa relative to *Ctsk*^{-/-}-vehicle exposed littermates (Fig. 5B).

We also measured the PDGF-BB concentration in bone marrow supernatants by ELISA. There was no significant difference of PDGF-BB concentration between *Ctsk*^{-/-}-vehicle treated mice (3.2 ± 0.3 ng/mL) and WT-vehicle treated mice (2.1 ± 0.6 ng/mL). PDGF-BB concentration was significantly decreased in WT-prednisolone mice (1.2 ± 1.4 ng/mL) relative with WT-vehicle treated littermates, whereas PDGF-BB was significantly increased in *Ctsk*^{-/-}-prednisolone mice (4.0 ± 4.3 ng/mL) relative to *Ctsk*^{-/-}-vehicle littermates (Fig. 5C). Overall, chronic high daily exposure to glucocorticoids in young WT mice results in decreased osteoclast numbers and PDGF-BB production. On the contrary, chronic high daily exposure to glucocorticoids in young *Ctsk*^{-/-} mice resulted in increased and unchanged osteoclast numbers and PDGF-BB production in the primary and secondary spongiosa, respectively.

Type H blood vessels and osteoblasts are maintained in young mice exposed to chronic daily high dose glucocorticoids with genetic deletion of *Cathepsin K*

We previously found that PDGF-BB secretion from TRAP⁺ cell couples type H vessel formation with bone formation in both bone modeling and remodeling [10]. Type H vessels are defined by high immunofluorescence for CD31 and endomucin (CD31^{hi}Emcn^{hi}) [29]. We thus explored the effect of prednisolone on CD31^{hi}Emcn^{hi} vessels and osteoblasts in *Ctsk*^{-/-} and WT mice. In regards to type H vessels, we observed that the majority of type H vessels are located in the primary spongiosa (43.4 ± 0.4 CD31^{hi}Emcn^{hi} cells per mm²) relative to secondary spongiosa in WT mice (12.7 ± 2.2 CD31^{hi}Emcn^{hi} cells per mm²) (Fig. 6A). Prednisolone treatment in WT mice resulted in a significant decrease in the number of CD31^{hi}Emcn^{hi} cells in both the primary and secondary spongiosa relative to vehicle (Fig. 6A–C). *Ctsk*^{-/-} mice at 6 weeks of age did not demonstrate a difference in type H vessels relative to WT vehicle mice, with the exception of *Ctsk*^{-/-}-prednisolone mice that had an increased number of CD31^{hi}Emcn^{hi} cells in primary spongiosa (Fig. 6A–B). Overall, *Ctsk*^{-/-} mice treated with chronic high dose prednisolone were resistant to the decreased type H vessel effect noted in WT mice on high dose prednisolone.

In regards to mature osteoblast numbers, similar to prior reports in adult mice [30], we noted that young WT mice treated with high doses of prednisolone had decreased osteocalcin-positive (Ocn⁺) cell numbers relative to WT-vehicle littermates in both primary and secondary spongiosa (Fig. 7A–B). At 6 weeks of age, there was no difference in the number of Ocn⁺ cells between WT-vehicle and *Ctsk*^{-/-}-vehicle mice (primary spongiosa: $P = 0.073$; secondary spongiosa: $P = 0.058$) (Fig. 7A, B). However, *Ctsk*^{-/-}-prednisolone mice had increased numbers of Ocn⁺ cells in the primary spongiosa relative to all other groups, but no difference noted in secondary spongiosa except relative to WT-prednisolone (Fig. 7A–B). Overall, *Ctsk*^{-/-} mice treated with chronic high dose prednisolone maintained osteoblast numbers in the secondary spongiosa and had a paradoxically increased numbers of osteoblasts in the primary spongiosa.

Osteocytes are also known to express *Ctsk*, particularly during lactation [17, 31]. In our peripubertal, non-breeding WT and *Ctsk*^{-/-} mice, *Ctsk*^{-/-}-vehicle mice were noted to have increased numbers of osteocytes in both primary and secondary spongiosa relative to WT-vehicle littermates (Suppl. Fig. 2). Prednisolone has previously been reported to induce osteocyte differentiation and apoptosis [13, 17, 18], which we also observed with a decreased number of osteocytes in WT-prednisolone mice in the primary and secondary spongiosa relative to WT-vehicle. *Ctsk*^{-/-}-prednisolone mice had decreased osteocyte numbers in the primary spongiosa, but no change in the secondary spongiosa relative to *Ctsk*^{-/-}-vehicle littermates (Suppl. Fig. 2).

Cathepsin K inhibition prevents Glucocorticoid-Induced Osteoporosis

To determine if glucocorticoid impairment of bone vasculature could be prevented in vivo by inhibition of cathepsin K, we treated WT mice with both prednisolone and a cathepsin K inhibitor (L-235) daily from 2–6 weeks of age. There was no significant difference in body weight among the groups (Suppl. Fig. 3A). The number of Trap⁺ cells (Fig. 8A–B), Trap and PDGF-BB co-positive cells (Fig. 8C–D), type H vessels as indicated by staining highly

positive for both CD31 and endomucin (Fig. 8E–F), and osterix⁺ cells (Fig. 8G–H) in the primary spongiosa were maintained when WT mice were co-administered prednisolone and L-235 relative to vehicle mice. Overall, microcomputed tomography analysis demonstrated no significant change in bone volume, trabecular number, trabecular space, or trabecular thickness in WT-prednisolone + L-235 mice relative to WT-vehicle littermates (Fig. 8I–J, Suppl. Fig. 3B).

PDGF-BB augments VEGF signaling in EPCs to preserve endothelial tube formation

To explore potential mechanisms how increased PDGF-BB signaling could prevent glucocorticoid-suppression to type H vessels, we performed Matrigel tube formation assay. Tube formation was decreased in EPCs treated with prednisolone relative to vehicle, enhanced by PDGF-BB, and preserved with co-treatment of prednisolone and PDGF-BB relative to vehicle (Fig. 9A–B). Quantitative RT-PCR confirmed down-regulation of genes promoting angiogenesis (VEGF) and osteogenesis (COX2, PGE2, Ezh2), with a likely compensatory increase in VEGFR2 (Fig. 9C). PDGF-BB increased VEGF gene expression, but decreased VEGFR2, TSP-1, PDGFR- β , and Ezh2 (Fig. 9C). Combination of PDGF-BB and prednisolone ameliorated changes in VEGF and VEGFR2, but decreased angiostatin, PDGFR- β , and Ezh2 (Fig. 9C). Overall, the changes in VEGF expression was similar to change in tube length when EPCs were exposed to prednisolone, PDGF-BB, or both prednisolone and PDGF-BB.

Discussion

Glucocorticoids are a mainstay of treatment in many pediatric disorders and are associated with a high prevalence of osteoporosis, ranging from 29–45% [12]. The clinical criteria for the diagnosis of GIO includes chronic exposure to glucocorticoids and a significant fracture history, regardless of bone mineral density (BMD) as a low BMD is not always observed with the skeletal fragility [32]. Although GIO in children is increasingly recognized, no evidence-based guidelines for prevention or treatment exist in regards to children who require long-term glucocorticoid therapy [33]. In the present study, we developed a young GIO mouse model to begin to elucidate the pathophysiology of GIO in the growing skeleton. We found that chronic high doses of prednisolone resulted in decreased osteoclasts, osteoblasts, and blood vessels in both the primary and secondary spongiosa. *Ctsk*^{-/-} mice were resistant to the reduction in bone volume with high dose chronic glucocorticoid exposure. *Ctsk*^{-/-} prednisolone-mice maintained osteoclast PDGF-BB production, which was associated with preservation of type H blood vessels and osteoblasts in the secondary spongiosa and, interestingly, increased in the primary spongiosa.

A major difference in the skeleton of young and adult mice is growth and acquisition of bone mass. Therefore, to further understand the spatial and temporal patterns of bone resorption and formation, we first evaluated how the number of osteoclasts and osteoblasts change during the rapid growth phase of mice from 2 to 8 weeks of age. In the primary spongiosa, the number of TRAP⁺ cells remained similar, while Osx⁺ cells decreased between 2, 4 and 8 weeks of age in wild type mice. This observation is consistent with a faster rate of growth and high bone turnover in young mice, declining with age. Genetic loss

of *Ctsk*, resulted in fewer TRAP⁺ cells in the younger mice (2 and 4 week old), but no differences in *Osx*⁺ cells until later age (8 weeks of age) in the primary spongiosa. Therefore, at the level of the primary spongiosa where modeling occurs, loss of cathepsin K activity appears to have predominantly anti-resorptive effects in younger mice (2–4 weeks) with anabolic effects during pubertal growth (4–8 weeks). In the secondary spongiosa, the number of TRAP⁺ cells in the secondary spongiosa were similar at younger ages (2–4 weeks) in wild type and *Ctsk*^{-/-} mice, declining between 4–8 weeks in both mice, but less so in *Ctsk*^{-/-} mice. *Osx*⁺ cells remained similar at all time points in WT secondary spongiosa, but were greater at young (2 week) and later (8 week age) ages in *Ctsk*^{-/-} mice relative to WT mice. Therefore, at the level of the secondary spongiosa where remodeling occurs, loss of cathepsin K activity appears to have both anti-resorptive and anabolic effects during early postnatal growth. The reason for the fluctuation in number of *Osx*⁺ cells may be related to preprogrammed cellular senescence and rapid growth that occurs immediately post-natally and again during the pubertal growth spurt [11]. If genetic loss of cathepsin K effects cell senescence remains unknown.

The effects of glucocorticoids on bone remodeling have been extensively studied in adult mice [4, 18]. The direct effect of glucocorticoids on osteoclasts appear to contribute significantly to skeletal fragility [14, 15, 34]. In vitro studies have previously found that murine osteoclast cultures exposed to glucocorticoids led to prolonged baseline survival of osteoclasts [16], while in vivo adult mouse studies have reported a transient increase in osteoclast numbers, followed by a decrease with more prolonged treatment [13, 16]. In our young GIO mouse model, we also noted a decrease in osteoclast numbers with chronic glucocorticoid exposure in WT mice. *Ctsk*^{-/-} prednisolone-mice maintained similar numbers of TRAP⁺ cells relative to *Ctsk*^{-/-} vehicle-mice and were resistant to steroid induced bone volume loss in the secondary spongiosa. As cathepsin K is expressed mainly in osteoclasts, our results suggest that osteoclasts are required for glucocorticoid-mediated inhibition of osteoblastogenesis, even in the last phase of GIO in young, growing mice. This is in contrast to the prevailing notion that the late phase of GIO in adult mice is mediated through osteoblasts in a cell autonomous manner.

The effect of glucocorticoids on modeling has not been previously explored. In our young GIO model, we found that cell response to glucocorticoids in *Ctsk*^{-/-} mice was different in the primary versus secondary spongiosa. Specifically, whereas osteoclast and osteoblast numbers, as well as type H blood vessels and PDGF-BB expression was unchanged in *Ctsk*^{-/-} mice treated with prednisolone relative to vehicle in the secondary spongiosa, all factors were increased in the primary spongiosa. Previous groups have also observed a difference in response of cells in primary and secondary spongiosa. For example, Wang et al found that PTH effect on bone volume/tissue volume was dependent on intact IGF-1R in the primary spongiosa, but independent of IGF1R in the secondary spongiosa [27]. Kameda et al found that sialic acid binding Ig-like lectin (Siglec)-15 deficient mice, which have defective osteoclast development and resorptive activity due to cytoskeletal dysregulation, maintain similar number of multinucleated osteoclasts in the primary spongiosa, but are decreased in the secondary spongiosa relative to WT mice [26]. Cathepsin K gene expression and protein have been shown to increase in bone following glucocorticoid treatment [17, 35]. Perhaps

the high number of osteoclasts in the primary spongiosa in young mice amplifies this response to glucocorticoids.

Fracture risk with glucocorticoid therapy increases prior to a recognized decline in BMD [18]. Our present study and several others have shown a reduction in bone vasculature in various GIO models [19, 24, 36–39]. As an adequate blood supply is vital for bone health [40], the effect of glucocorticoids on vasculature may be one additional explanation for bone fragility. Regarding the mechanism of the reduction in vasculature, glucocorticoids have been shown to suppress hypoxia-inducible factor 1- α transcription [39, 41], reduce vascular endothelial growth factor (VEGF) [18, 24, 36–38, 42] and increase thrombospondin-1 [43]. Conjugation of alendronate to a novel peptidomimetic (LLP2A) to integrin $\alpha_4\beta_1$ expressed on mesenchymal stem cells prevented decline of VEGF-A and bone vasculature in a 4-month old prednisolone mouse model [42]. In our young GIO mouse model, we investigated a specific type of blood vessel associated with osteogenesis, described as Type H vessels, which can be distinguished by high immunofluorescence signaling for CD31 and Emcn [29, 44]. We noted type H vessels were more abundant in the primary relative to secondary spongiosa, both of which were reduced in response to high dose chronic prednisolone in WT mice. *Ctsk*^{-/-} prednisolone-mice had increased and similar quantities of type H vessels in primary and secondary spongiosa, respectively. We have previously reported that genetic loss of cathepsin K increases preosteoclast secretion of PDGF-BB to couple type H angiogenesis to osteogenesis [10]. We observed that *Ctsk*^{-/-} prednisolone-mice had increased PDGF-BB and TRAP co-staining in the primary spongiosa, which were maintained in the secondary spongiosa relative to all other groups. Bone marrow PDGF-BB concentrations were decreased in WT prednisolone-mice, but were increased in *Ctsk*^{-/-} prednisolone-mice relative to respective vehicle controls, which may serve as a potential biomarker for GIO. We found that increasing PDGF-BB concentrations can overcome the prednisolone-induced decline in endothelial tube formation, at least partially by augmenting endothelial cell VEGF production.

GIO is a significant problem for both children and adults, remaining the leading cause of iatrogenic and secondary osteoporosis [45]. BMD underestimates the risk of fracture in GIO [18] and often is not diagnosed until an incident vertebral compression fracture is noted [46]. Further studies are required, both clinically and preclinically, on the complex actions of glucocorticoids, particularly in relation to the underlying disease and stage of skeletal maturity and variable effects on bone modeling and remodeling. The use of a cathepsin K inhibitor may be a potential preventative treatment strategy for children with GIO, particularly with the strong efficacy as reported in odanacatib clinical trials of post-menopausal osteoporosis [47–50]. However, additional preclinical studies would be needed given the concern of potential adverse side effects that have limited further drug development of odanacatib in post-menopausal osteoporosis [51]. Other potential targets to consider could include other factors made specifically by osteoclasts that do not induce osteoclast apoptosis, such as DC-STAMP or Siglec-15 inhibitors [52, 53]. Safe, effective therapies may lower intervention thresholds, allowing for prevention rather than treatment of GIO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Loss of cathepsin K expression does not significantly alter osteoblast lineage cells or bone volume in primary spongiosa until late puberty in mice.

(A-D) Representative tartrate-resistant acid phosphatase (TRAP) staining (pink) (A) and Osterix (Osx) staining (brown) images (C) and quantitative analysis in tibia primary spongiosa of osteoclast number per trabecular bone area (N.Oc/Tb.Ar) (B) and Osx-positive cells per trabecular bone area (Osx⁺ cells/Tb.Ar) (D) of WT and *Ctsk*^{-/-} mice. Black arrows point to Osterix⁺ cells. (E) Quantitative analysis of tibia trabecular cancellous bone area per tissue area (Tb. Ar/T. Ar) in primary spongiosa of 2, 4, and 8 week old *Ctsk*^{+/-} wild type (WT) and *Ctsk*^{-/-} mice. Hematoxylin counterstained nuclei purple. Scale bar, 50 µm. n = 3 per group. Data shown as mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2. Secondary spongiosa osteoblast lineage cells and bone volume are increased throughout the postnatal growth spurt in *Ctsk*^{-/-} mice.

(A-D) Representative tartrate-resistant acid phosphatase (TRAP) staining (pink) (A) and Osterix (Osx) staining (brown) images (C) and quantitative analysis in tibia secondary spongiosa of osteoclast number per trabecular bone area (N.Oc/Tb.Ar) (B) and Osx-positive cells per trabecular bone area (Osx⁺ cells/Tb.Ar) (D) of WT and *Ctsk*^{-/-} mice. Black arrows point to Osterix⁺ cells. (E) Quantitative analysis of the trabecular cancellous bone area per tissue area (Tb. Ar/T. Ar) of tibia secondary spongiosa in 2, 4, and 8 week old *Ctsk*^{+/+} wild type (WT) and *Ctsk*^{-/-} mice. Hematoxylin counterstained nuclei purple. Scale bar, 50 µm. n = 3 per group. Data shown as mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3. Deletion of *Ctsk* prevents prednisolone associated bone loss

(A) Representative microcomputed tomography results of distal femur of 6 week old *Ctsk*^{+/+} wild type (WT) and *Ctsk*^{-/-} mice injected with either vehicle (veh) or prednisolone 10 mg/m²/day (pred) for 4 weeks. (B-E) Quantitative analysis of trabecular bone volume per tissue volume (BV/TV) (B), trabecular number (Tb. N) (C), trabecular separation (Tb. Sp) (D) and trabecular thickness (Tb. Th) (E) of WT and *Ctsk*^{-/-} mice injected with either vehicle or prednisolone. n = 6 per group. Data shown as mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4. TRAP⁺ cells are increased in *Ctsk*^{-/-} mice regardless of exposure to vehicle or prednisolone.

(A-B) Representative tartrate-resistant acid phosphatase (TRAP) staining (pink) images and quantitative analysis of the number of TRAP positive cells (TRAP⁺) in femoral primary (A) and secondary spongiosa (B) of 6 week old *Ctsk*^{+/+} wild type (WT) and *Ctsk*^{-/-} mice injected with either vehicle (veh) or prednisolone 10 mg/m²/day (pred) for 4 weeks. Hematoxylin counterstained nuclei purple. Scale bar, 20 μ m. n = 6 per group. Data shown as mean $\pm$ s.d. *P < 0.05, **P < 0.01.

Figure 5. PDGF-BB is increased in *Ctsk*^{-/-} prednisolone-exposed mice.

(A-B) Representative images of immunofluorescence staining of platelet-derived growth factor type BB (PDGF-BB) (green) and tartrate-resistant acid phosphatase (TRAP) (red) with quantification of number of PDGF-BB⁺ TRAP⁺ cells (yellow) in femoral primary (A) and secondary spongiosa (B) of 6 week old *Ctsk*^{+/+} wild type (WT) and *Ctsk*^{-/-} mice injected with either vehicle (veh) or prednisolone 10 mg/m²/day (pred) for 4 weeks. DAPI stains nuclei blue. Scale bar, 50 μ m. (C) Bone marrow PDGF-BB concentration as analyzed by ELISA. n = 6 per group. Data shown as mean $\pm$ s.d. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6. Type H endothelial CD31^{hi}Emcn^{hi} cells are reduced in wild type, but maintained in *Ctsk*^{-/-} prednisolone-exposed mice.

(A) Representative images of CD31 (red) and endomucin (Emcn) (green) immunostaining of CD31^{hi}Emcn^{hi} (yellow) cells in distal femur metaphysis of 6 week old *Ctsk*^{+/+} wild type (WT) and *Ctsk*^{-/-} mice injected with either vehicle (veh) or prednisolone 10 mg/m²/day (pred) for 4 weeks. Red box outlines region of interest with magnification of primary (top panels) and secondary (bottom panels) spongiosa. DAPI stains nuclei blue. Scale bar, 50 μ m. (B-C) Quantification of CD31^{hi}Emcn^{hi} (yellow) cells per mm² in primary (B) and secondary spongiosa (C). n = 6 per group. Data shown as mean $\pm$ s.d. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7. Osteoblasts are increased in primary spongiosa and maintained in secondary spongiosa of *Ctsk*^{-/-} prednisolone-exposed mice

Representative osteocalcin staining (brown) images and quantitative analysis of the number of osteocalcin-positive cells (OCN⁺) in femoral primary (A) and secondary spongiosa (B) of 6 week old *Ctsk*^{+/+} wild type (WT) and *Ctsk*^{-/-} mice injected with either vehicle (veh) or prednisolone 10 mg/m²/day (pred) for 4 weeks. Hematoxylin counterstained nuclei purple. Scale bar, 50 μ m. n = 6 per group. Data shown as mean $\pm$ s.d. *P < 0.05, **P < 0.01.

Figure 8. L-235 protects bone from glucocorticoid induced bone loss

(A-B) Representative tartrate resistance acid phosphatase (Trap) (magenta) (A) and quantification of number of positively stained cells per mm² (B) in distal femoral primary spongiosa of 6 week old wild type mice injected with either vehicle, prednisolone 10 mg/m²/day (Pred), or prednisolone plus the cathepsin K inhibitor L-235 20 mg/kg daily (Pred + L-235) for 4 weeks. Trap counterstained with toluidine blue. Scale bar, 50 μ m. (C-D) Immunofluorescence staining (C) of Trap (red) and platelet-derived growth factor type BB (PDGF-BB) (green) with quantification of number of TRAP⁺PDGF-BB⁺ cells (yellow) (D). DAPI stains nuclei blue. Scale bar, 100 μ m. (E-F) Immunofluorescence staining (E) and quantification (F) of CD31 (red) and endomucin (Emcn) (green) with quantification of vessel surface per mm squared. Co-stained cells appear yellow. Scale bar, 100 μ m. n = 6 per group. (G-H) Osterix (Osx) immunohistochemical staining (nuclei stains brown) (G) and quantification of number of positively stained cells per mm² (H). Counterstained with hematoxylin. Scale bar, 20 μ m. (I-J) Representative microcomputed tomography (I) and quantitative analysis (J) of bone volume per tissue volume (BV/TV). Scale bar, 1 mm. n = 6

per group. Data shown as mean $\pm$ s.d. *P < 0.05, **P < 0.01, relative to vehicle. #P < 0.05, ##P < 0.01, ###P < 0.001 relative to Pred.

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Figure 9. PDGF-BB augments prednisolone-suppression of endothelial tube formation via VEGF signaling

(**A-B**) Representative Matrigel tube formation assay image (**A**) and quantification (**B**) of tube length of endothelial precursor cells (EPCs) cultured with 1X PBS (Control), 60 ng/mL PDGF-BB alone, 10^{-6} M Prednisolone (Pred) alone, or PDGF-BB plus Prednisolone for 4 hours. Scale bar, 200 μ m. (**B**) Relative gene expression of denoted mRNA by RT-PCR of EPCs harvested from tube formation assays. Experiments replicated 3 times. Data shown as mean $\pm$ s.d. * $P < 0.05$, *** $P < 0.001$ comparing control relative to Pred, & $P < 0.05$, && $P < 0.01$ comparing control relative to PDGF-BB, and ## $P < 0.01$ comparing control relative to Pred + PDGF-BB.