Osteoblast CFTR Inactivation Reduces Differentiation and Osteoprotegerin Expression in a Mouse Model of Cystic Fibrosis-Related Bone Disease

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Abstract

Low bone mass and increased fracture risk are recognized complications of cystic fibrosis (CF). CF-related bone disease (CFBD) is characterized by uncoupled bone turnover—impaired osteoblastic bone formation and enhanced osteoclastic bone resorption. Intestinal malabsorption, vitamin D deficiency and inflammatory cytokines contribute to CFBD. However, epidemiological investigations and animal models also support a direct causal link between inactivation of skeletal cystic fibrosis transmembrane regulator (CFTR), the gene that when mutated causes CF, and CFBD. The objective of this study was to examine the direct actions of CFTR on bone. Expression analyses revealed that CFTR mRNA and protein were expressed in murine osteoblasts, but not in osteoclasts. Functional studies were then performed to investigate the direct actions of CFTR on osteoblasts using a CFTR knockout (Cftr−/−) mouse model. In the murine calvarial organ culture assay, Cftr−/− calvariae displayed significantly less bone formation and osteoblast numbers than calvariae harvested from wildtype (Cftr+/+) littermates. CFTR inactivation also reduced alkaline phosphatase expression in cultured murine calvarial osteoblasts. Although CFTR was not expressed in murine osteoclasts, significantly more osteoclasts formed in Cftr−/− compared to Cftr+/+ bone marrow cultures. Indirect regulation of osteoclastogenesis by the osteoblast through RANK/RANKL/OPG signaling was next examined. Although no difference in receptor activator of NF-kB ligand (Rankl) mRNA was detected, significantly less osteoprotegerin (Opg) was expressed in Cftr−/− compared to Cftr+/+ osteoblasts. Together, the Rankl/Opg ratio was significantly higher in Cftr−/− murine calvarial osteoblasts contributing to a higher osteoclastogenesis potential. The combined findings of reduced osteoblast differentiation and lower Opg expression suggested a possible defect in canonical Wnt signaling. In fact, Wnt3a and PTH-stimulated canonical Wnt signaling was defective in Cftr−/− murine calvarial osteoblasts. These results support that genetic inactivation of CFTR in osteoblasts contributes to low bone mass and that targeting osteoblasts may represent an effective strategy to treat CFBD.

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Introduction

Advances in cystic fibrosis (CF) care in the past 20 years have dramatically extended survival of CF patients, but age related complications such as low bone mass have emerged. The association of low bone mineral density (BMD) with CF was first reported in 1979 [1,2]. In a large study of adult CF patients, 57% had osteoporosis that translated to a 100-fold greater risk of vertebral compression fracture [3]. Fractures not only cause pain and disability, but vertebral fractures and kyphosis further impair breathing biomechanics and often disqualify a patient from lung transplantation [4]. CF-related bone disease (CFBD) is the result of decreased osteoblast-dependent bone formation and increased osteoclastic bone resorption. This pattern of uncoupled bone turnover has been reported in both animal and clinical studies [5–9].

CFBD has been attributed to vitamin D deficiency, poor nutrition, hypogonadism, increase in inflammatory cytokines and glucocorticoid therapy. Other risk factors include male gender, advanced lung disease, malnutrition, and low fat-free body mass [10]. The F508del-CFTR (cystic fibrosis transmembrane conductance regulator) is the most frequent mutation that results in non-delivery of the protein to the cell membrane and confers an essentially null CFTR protein. This mutation compared to less severe CFTR mutations was also independently associated with reduced BMD in adult CF patients [10]. Whether these risk factors contribute indirectly to bone loss or CFTR mutations directly impact bone, has been a topic of controversy [11].

The skeletal phenotype of CF mouse models suggests that CFTR indeed has direct actions on bone. An advantage of CF mouse models in studying bone is the lack of CF-related...
complications found in human CF patients that influence BMD. CFTR knockout mice do not develop significant lung disease or a secondary increase in circulating inflammatory cytokines common in CF patients [9,12,13]. Proposed mechanisms for the lack of profound lung disease in CF mouse models include alternative chloride channels present in the mouse airway that compensate for CFTR absence and differences in bacterial airway colonization [14,15]. Exocrine pancreatic insufficiency also does not occur in CF mouse models. Similarly, other non-CFTR chloride channels present in pancreatic ducts and acini may prevent insufficiency of the mouse exocrine pancreas and subsequent malnutrition, vitamin D deficiency and secondary hyperparathyroidism [14].

Although intestinal obstruction occurs in both humans and mice with defective CFTR, this alone would not contribute directly to CFBD. Despite the absence of these confounding factors, both CFTR knockout and F508del-CFTR mouse models recapitulate the human CFBD phenotype of reduced trabecular bone volume and cortical thickness, and impaired bone formation and enhanced bone resorption [8,9,12,16]. Similarly, “gut corrected” CFTR knockout mice also demonstrate a similar bone phenotype further suggesting that CF bone disease is not solely due to intestinal obstruction [17].

We examined CFTR expression and determined the direct biological consequences of CFTR inactivation in murine bone using the knockout mouse model. CFTR was expressed in the osteoblast and inactivation resulted in defective differentiation, delayed new bone formation, and impaired osteoblast canonical Wnt signaling. Although CFTR was not expressed in osteoclasts, enhanced osteoclastogenesis is likely a direct effect of reduced osteoblast osteoprotegerin expression. This is the first report demonstrating direct in vitro effects of CFTR on osteoblasts. Defining the mechanisms of bone loss and determining optimal treatments are critical because CF patients are now living longer and the prevalence of fractures is expected to increase.

Materials and Methods

Animal use

Animal experimentation was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Study mice with a global CFTR inactivation have been described previously [18,19]. The colony was maintained by breeding Cfr+/− to Cfr+/+ C57BL/6 mice. Cfr−/− mice were generated by crossing Cfr+/− to Cfr+/− mice.

Murine calvarial organ and primary osteoblast cultures

Calvariae were harvested from Cfr−/− and Cfr+/+ pups or from pups derived from WT timed-pregnant ICR Swiss female mice as previously described [20,21]. Briefly, four-day-old pups were euthanized by CO2 inhalation. Calvariae were excised, cut along the sagittal suture, and each half placed on a stainless steel grid in a 12-well tissue culture plate containing 1 ml BGJ media (Life Technologies), 0.1% bovine serum albumin, 100 IU/ml penicillin and 100 μg/ml streptomycin. After 14 days in culture, calvariae were fixed in 10% buffered formalin for 24 hours and decalcified with 10% EDTA for 48 hours. Tissue samples were then processed, paraffin-embedded, sectioned and stained with H&E/Orange-G. New bone area and osteoblast number were analyzed in quadruplicate samples using the BioQuant Image Analysis Software (BIOQUANT Image Analysis Cooperation, Nashville, TN). Histomorphometric analysis of calvariae was performed in quadruplicate using a 20X objective lens. New bone area and osteoblast number was determined from a calvarial microscopic field that measured 0.57 mm in length.

For primary osteoblast cultures, calvariae were washed in PBS and then placed in PBS (2 ml per mouse litter) containing 0.1% collagenase (Wako Pure Chemical Industries) and 0.2% dispase (Roche Applied Science). Calvariae were agitated at 37°C for seven minutes to release cells. The first extraction was discarded and the process was repeated three times. Osteoblasts isolated in the last three extractions were combined [22]. Cells were plated at a density of 10⁶ cells/ml in αMEM, 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

For osteoblast differentiation assays, primary osteoblast cultures were prepared from Cfr−/− and Cfr+/+/− calvariae. Seven days after confluence, cells were washed, fixed in formalin for 30 minutes, washed again and stained for alkaline phosphatase using SigmaFast BCIP/NTB reagent (Sigma-Aldrich).

Proliferation of Cfr−/− and Cfr+/+ calvarial osteoblasts was assessed by bromodeoxyuridine (BrdU) staining. Briefly, 0.5×10⁴ Cfr−/− and Cfr+/+ calvarial osteoblasts were plated into each well of 96-well plate in quadruplicate. Three days after plating, BrdU was added according the manufacturer’s directions (Roche) and proliferation was determined by colorimetric assessment. Apoptosis was determined using a FITC-annexin V cell membrane detection kit (BD Pharmingen) with FACS analysis.

Murine bone marrow osteoclast cultures

Bone marrow was collected from femurs and tibias of 8–9 week old Cfr−/− and Cfr+/+ mice. Bone marrow cells were counted using a Coulter Z2 Particle Count and Size Analyzer (Beckman Coulter, Inc), excluding cells less than 7.5 μm in diameter. Cells were plated in quadruplicate at a density of 2×10⁶ cells/0.5 ml in each well of a 24-well plate. Bone marrow isolates were cultured in α-MEM containing L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum and 1×10⁻⁸ M 1,25(OH)₂D₃. Osteoclast cultures were stained on day 8 with a tartrate-resistant acid phosphatase (TRAP) kit (Sigma) and osteoclasts, TRAP-staining cells containing ≥3 nuclei, were counted via visual inspection by light microscopy.

Immunohistochromistry

Calvariae were harvested from 4 day-old mouse litter containing Cfr−/− and Cfr+/+ pups. After confirmation of genotype, Cfr−/− and Cfr+/+ calvariae were fixed in 10% formalin and decalcified. Calvariae were processed, paraffin embedded and sectioned. Sections were blocked with 10 mg/ml BSA in PBS and incubated overnight at 4°C with the anti-CFTR 3G11 rat monoclonal antibody (Cystic Fibrosis Folding Consortium) at a 1:500 dilution. After washing with PBS, sections were incubated with an anti-rat biotin-conjugated secondary antibody for 30 minutes. Vectastain ABC Reagent (Vector Laboratories) was used for detection. Sections were counterstained with hematoxylin.

Immunofluorescence

Three-week-old Cfr−/− and Cfr+/+ bone marrow from tibiae, femora and humerus was flushed with αMEM, centrifuged, and plated at a density of 6×10⁵ cells/cm² on collagen-coated cover slips in αMEM, 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. On day two, 10 ng/ml M-CSF (R&D Systems) and 30 ng/ml RANKL (R&D Systems) were added. On day 8, cells were fixed in 4% paraformaldehyde for 20 min at RT, washed with PBS and permeabilized with 0.1% Triton X-100 for 15 min at RT. After blocking with 10 mg/ml BSA (Sigma-Aldrich) in PBS, cells were incubated overnight at 4°C with the anti-CFTR 3G11 rat monoclonal (1:500 dilution, Cystic Fibrosis Folding Consortium), and/or 3G11 and anti-
NHERF (1:500, Affinity Bioreagents) followed by incubation for 1 hour with Alexa 555 anti-rat IgG (1:500, Life Technologies), and/or Alexa 488 anti MOUSE IgG (1:500, Life Technologies), and 4’,6-diamidino-2-phenylindole (DAPI) (300 nM, Life Technologies). Images were obtained using a Zeiss LSM 710 Laser confocal microscope system.

Real-time PCR

Primary osteoblast cultures were washed in ice-cold PBS and RNA was extracted using the RNeasy Kit (Qiagen). A DNase digestion step was included during RNA purification. Real-time PCR (RT-PCR) was performed using the iQ SYBR Green Supermix and MyIQ Single-Color Real-Time PCR Detection System (BioRad). Changes in mRNA concentration were determined using the 2^-A^Ct method and RpL32 as the internal control. Primer sequences were: Cfr F: ctcagtcgcagcgttc, R: gaccaaatcagcactagca; Pth F: cagggtgcggagaaggttcaaggg, R: cttagggcactggtgaaacta; Rankl F: tgcaccttaggcaggctag, R: ecca- cacatgtgttgcagttc. Rankl and Opg expression was assessed after 24 hours of human parathyroid hormone (PTH) (1–34) 10 nM (American Peptide) treatment.

Statistical analyses

Statistical analyses were performed using Prism 5.0 software. Comparisons of two groups were performed using an unpaired, two-tailed t test. Comparisons of three or more groups were performed using one-way ANOVA with Tukey post-test analysis. Significant differences are indicated (*p<0.05; **p<0.01; ***p<0.001).

Results

CFTR expression in bone

Initial studies were directed at determining CFTR expression in mouse bone cells. Cfr mRNA was detected in calvarial osteoblasts as well as murine liver, lung, and pancreas (Figure 1A). Comparable pancreas and osteoblast mRNA concentrations suggest that the degree of Cfr expression in the osteoblast was physiologically relevant since Cfr inactivation also results in pancreatic disease. In vivo protein expression was next examined utilizing neonatal murine calvarial organ cultures, a rich source of osteoblasts. CFTR protein was detected in wild type (+/+) calvariae by immunostaining using the 3G11 monoclonal anti-CFTR antibody (Figure 1B). Osteoblasts and osteocytes embedded within bone displayed similar expression. In a recent report, the 3G11 antibody displayed excellent CFTR detection specificity compared to other antibodies that reportedly detect mouse CFTR in a panel of Cftr murine calvarial osteoblasts revealed abundant punctate intracellular staining (Figure 1C).

Osteoclast Cfr expression was next examined. Cfr mRNA was not detected in the mouse macrophage cell line RAW 264.7 (Figure 2A). These osteoclast precursor cells were then treated with macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) to induce osteoclastogenesis. Message for TRAP, a specific osteoclast marker, was detected in treated, but not untreated, RAW 264.7 cells indicating successful osteoclast differentiation. Despite differentiation with M-CSF and RANKL, Cfr mRNA remained undetectable after 35 PCR cycles (Figure 2A). To confirm the absence of expression, CFTR protein was not detected in osteoclasts derived from mouse bone marrow by immunofluorescence staining (Figure 2B). An antibody to the membrane protein Na+/H+ exchange regulatory cofactor-1 (NHERF) served as a control for osteoclast membrane staining.

CFTR osteoblast actions

The consequence of restricted osteoblast CFTR expression in bone was investigated. The murine calvarial organ culture assay is a reliable method to measure osteoblast activity and bone formation [20,21]. Calvariae were harvested from Cfr+/– and Cfr/+ 4 day-old pups and the calvarial organ culture bone formation assay was performed. After 14 days in culture, Cfr+/– calvariae displayed significantly less new bone (6430 ± 16440 µm², p = 0.0023) and fewer osteoblasts (109 ± 194 Obl/24 mm², p = 0.04) than Cfr+/+ calvariae (Figure 3A). The in vitro effects of osteoblast CFTR inactivation were also examined. Calvarial osteoblasts harvested from Cfr+/- and Cfr–/– littermates were plated at the same density and grown in cell culture for seven days after achieving confluence. Osteoblast cultures were stained for alkaline phosphatase, a marker of bone resorption. An equivalent amount of Message for TRAP, a specific osteoclast marker, was detected in treated, but not untreated, RAW 264.7 cells indicating successful osteoclast differentiation. Despite differentiation with M-CSF and RANKL, Cfr mRNA remained undetectable after 35 PCR cycles (Figure 2A). To confirm the absence of expression, CFTR protein was not detected in osteoclasts derived from mouse bone marrow by immunofluorescence staining (Figure 2B). An antibody to the membrane protein Na+/H+ exchange regulatory cofactor-1 (NHERF) served as a control for osteoclast membrane staining.

Indirect effects of CFTR on osteoclastogenesis

Despite the lack of CFTR osteoclast expression, CF patients and CF animal models demonstrate enhanced osteoclastic bone resorption. An in vitro osteoclastogenesis assay was performed on bone marrow harvested from Cfr+/- and Cfr–/– mice. This osteoclast formation assay relies on the presence of bone marrow-derived osteoblasts and stromal cells to provide M-CSF and RANKL and to drive osteoclastogenesis. Significantly more TRAP-positive osteoclasts were found in Cfr–/– bone marrow cultures compared to cultures harvested from Cfr+/- mice (Figure 4A).

Critical signaling molecules that regulate osteoclastogenesis are RANKL and Osteoprotegerin (OPG). OPG, a member of the tumor necrosis factor (TNF) receptor superfamily, is a secreted RANKL decoy receptor of osteoblastic-lineage cells [24,25]. OPG achives its effect on osteoclasts indirectly by binding to, and blocking the effect of RANKL [26]. Therefore, the ratio of RANKL to OPG determines osteoclast formation potential.

The effects of CFTR inactivation on osteoblast Rankl and Opg expression were examined to uncover mechanisms of enhanced bone resorption. Subconfluent Cfr+/- and Cfr–/– osteoblasts were treated with and without PTH (1–34) for 24 hours, and Rankl and Opg mRNA was measured (Figure 4B). PTH was used as a control to increase Rankl and decrease Opg osteoblast expression [24,27–29]. An equivalent amount of Rankl mRNA was detected in Cfr+/- and Cfr–/– osteoblasts. PTH (1–34) expectedly increased Rankl mRNA to a similar degree in both groups. However, CFTR inactivation led to significantly less Opg expression.
expression. PTH (1–34) did not alter Opg mRNA in Cftr−/− osteoblasts but expectedly reduced mRNA concentration in Cftr+/+ osteoblasts. Overall, the Rankl:Opg ratio was higher in Cftr−/− compared to Cftr+/+ osteoblasts with or without PTH (Figure 4B). These data suggested that increased osteoclastic bone resorption with CFTR inactivation in humans and mice may be due to enhanced osteoblast-mediated osteoclastogenesis. A follow-up experiment examined the durability of these data in osteoblasts cultured 7 and 14 days after reaching confluence. Opg expression remained significantly lower at 7 days in Cftr−/− osteoblasts, but did not reach significance at 14 days post-confluence (Figure 4C). No significant difference in Rankl between the Cftr+/+ and the Cftr−/− osteoblasts cultured for 7 or 14 days post-confluence was detected.

Cyclic AMP (cAMP) mediates PTH-stimulated reduction in Opg and increase in Rankl expression via coupling of the G-protein coupled receptor PTH1R to G-protein Gαs [28]. Differences in cAMP cellular accumulation with PTH in Cftr+/+ and Cftr−/− osteoblasts were measured. A brief 10 minute PTH (1–34) treatment increased cAMP approximately two-fold in both Cftr+/+ and Cftr−/− osteoblasts suggesting that the absence of osteoblast CFTR does not alter PTH-induced cAMP accumulation (Figure 5A).

Another critical signaling pathway that regulates both osteoblast differentiation and Opg expression is canonical Wnt signaling [30,31]. Forty-eight hours of Wnt3a and PTH treatment expectedly increased canonical Wnt activity in Cftr+/+ osteoblasts but not in Cftr−/− osteoblasts (Figure 5B). These data suggest that dysfunctional canonical Wnt signaling contributes to the uncoupled bone phenotype characteristic of CF.

Discussion

The average life expectancy of CF patients 60 years ago was less than 5 years, but treatment advances have extended the average life expectancy now to 36.8 years [32]. It is expected that continued progress in this area will result in even greater longevity. Because CF patients are now older, other age-related complications have emerged such as clinically significant fractures of the spine, hip and forearm [3]. Malnutrition, glucocorticoids, chronic inflammation and hypogonadism individually contribute to reduced bone accrual and low BMD in CF patients. Clinical observations and animal models of CF bone disease hint that CFTR mutation in bone also impacts bone remodeling [5–9]. The results reported here support a CF disease model in which CFTR expression in bone directs reduced osteoblast differentiation and enhanced osteoclastic bone resorption.

The most recognizable CF complications are the result of CFTR dysfunction in endoderm-derived secretory epithelial cells. This report and other recent studies demonstrate that CFTR is expressed in mesenchyme-derived osteoblasts, odontoblasts, chondrocytes and myocytes and that mutations directly perturb cellular

Figure 1. CFTR expression in osteoblasts. (A) Total RNA was harvested from mouse tissues. Cfr mRNA expression in murine primary osteoblast cultures, pancreas, liver and lung was assessed by real-time RT PCR. (B) Calvariae were harvested from Cfr+/+ and Cfr−/− 4-day-old pups, fixed, sectioned immunostained for CFTR using the 3G11 rat anti-CFTR monoclonal antibody and counterstained with hematoxylin. (C) Osteoblasts were prepared by digestion from murine calvariae and cultured on collagen-coated glass slides. CFTR (red) was identified by immunofluorescence using the 3G11 antibody. Nuclei (blue) were identified by DAPI staining. doi:10.1371/journal.pone.0080098.g001

Figure 2. Lack of CFTR expression in osteoclasts. (A) Cfr mRNA expression analysis of calvarial osteoblasts, undifferentiated and osteoclast-differentiated (M-CSF/RANKL-treated) RAW264.7 murine monocyte cells. Trap mRNA expression served as a control for osteoclast formation. (B) Immunofluorescence did not reveal CFTR (red) expression in murine bone marrow-derived multinucleated osteoclasts. Membrane-localized protein Na+/H+ exchange regulatory cofactor-1 (NHERF1) (green) served as a positive control for membrane staining. Nuclei (blue) was identified by DAPI staining. doi:10.1371/journal.pone.0080098.g002
Figure 3. CFTR inactivation reduced osteoblast differentiation and bone formation. (A) The murine calvarial organ culture assay was performed on Cftr+/+ and Cftr−/− mouse pups. After two weeks in culture, new bone formation and osteoblast number was significantly less with Cftr inactivation. Yellow arrows indicate the area of actively mineralizing bone indicated by orange G staining that is distinct from the red staining of eosin. (B) Murine calvarial osteoblasts harvested from Cftr+/+ and Cftr−/− calvariae were grown in culture. Seven days after reaching confluence, osteoblast differentiation was assessed by alkaline phosphatase staining. (C) Proliferation, as assessed by BrdU staining, was modestly increased in Cftr+/+ compared to Cftr−/− calvarial osteoblasts. (D) The proportion of calvarial osteoblasts that were viable, undergoing early apoptosis or late apoptosis was unchanged with CFTR inactivation. (* = p < 0.05; ** = p < 0.01)
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Figure 4. CFTR inactivation increased osteoclastogenesis and Rankl:Opg osteoblast expression. (A) Bone marrow was flushed from Cftr+/+ and Cftr−/− mice and osteoclast formation assays performed. Osteoclasts were identified by TRAP staining as cells containing ≥ 3 or more nuclei. More osteoclasts were found in Cftr−/− bone marrow. (B) Rankl and Opg mRNA expression was determined by real-time RT PCR in Cftr+/+ and Cftr−/− calvarial osteoblasts with and without PTH (1–34) 10 nM treatment for 24 hours. Overall, CFTR inactivation increased the Rankl:Opg ratio in both PTH (1–34)-treated and untreated osteoblasts. (C) In long-term osteoblast cultures, decreased Opg expression persisted post-confluence despite no significant difference in Rankl. Opg expression remained significantly lower at 7 days in Cftr−/− osteoblasts, but did not reach significance at 14 days post-confluence. (* = p < 0.05; ** = p < 0.01; *** = p < 0.001; NS = not significant)
doi:10.1371/journal.pone.0080098.g004
Significant results were observed, which suggest that CFTR inactivation may have additional effects on pathways outside of canonical Wnt signaling. The evidence provides strong support for the hypothesis that CFTR may also act as a scaffold protein to anchor other membrane proteins and assist in proper cell signaling.

Other investigators have also speculated that CFTR may also act as a scaffold protein to anchor other membrane proteins and assist in proper cell signaling. The evidence provides strong support for the hypothesis that CFTR may also act as a scaffold protein to anchor other membrane proteins and assist in proper cell signaling.

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Author Contributions

Conceived and designed the experiments: MSS GAC. Performed the experiments: MSS KLC VH CRM LKD WJC GAC. Analyzed the data: MSS VH GAC. Wrote the paper: MSS GAC.
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