Published in final edited form as:

Mol Cell Endocrinol. 2017 January 5; 439: 165–174. doi:10.1016/j.mce.2016.08.004.

Celecoxib treatment of fibrous dysplasia (FD) in a human FD cell line and FD-like lesions in mice with protein kinase A (PKA) defects

Emmanouil Saloustros¹, Sisi Liu¹, Edward L. Mertz², Nisan Bhattacharyya³, Matthew F. Starost⁴, Paraskevi Salpea¹, Maria Nesterova¹, Michael Collins³, Sergey Leikin², and Constantine A. Stratakis^{1,#}

¹Section on Endocrinology and Genetics (SEGEN), Program on Developmental Endocrinology & Genetics (PDEGEN), Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), Bethesda, MD 20892, USA

²Section on Physical Biochemistry, Office of the Scientific Director, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), Bethesda, MD 20892, USA

³Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research (NIDCR), NIH, Bethesda, MD 20892, USA

⁴Office of Research Services (ORS), Division of Veterinary Resources (DVR), Office of the Director (OD), National Institutes of Health (NIH), Bethesda, Maryland 20892, USA

Abstract

Osteochondromyxomas (OMX) in the context of Carney complex (CNC) and fibrous dysplasia (FD)-like lesions (FDLL) in mice, as well as isolated myxomas in humans may be caused by inactivation of *PRKARIA*, the gene coding for the type 1a regulatory subunit (R1 α) of cAMP-dependent protein kinase (PKA). OMXs and FDLL in mice lacking *Prkar1a* grow from abnormal proliferation of adult bone stromal cells (aBSCs). *Prkar1a* and *Prkaca* (coding for C α) haploinsufficiency lead to COX2 activation and prostaglandin E2 (PGE2) production that, in turn, activates proliferation of aBSCs. Celecoxib is a cyclooxygenase-2 (COX2) inhibitor. We hypothesized that COX-2 inhibition may have an effect in FD and FDLL. *In vitro* treatment of a human cell line prepared from a FD patient with Celecoxib resulted in decreased PGE2 and cell proliferation. Treatment of mice haploinsufficient for R1 α and C α with 1,500 mg/kg Celecoxib led to decreased PGE2 and proliferation and increased apoptosis, with a corresponding gene expression profile, resulting in dramatic reduction of tumor growth. Furthermore, the treatment

[#]Corresponding author: Constantine A. Stratakis, M.D., D(Med)Sci., SEGEN, PDEGEN, NICHD, NIH, 10 Center Drive, Building 10, NIH-Clinical Research Center, Room 1-3330, MSC1103, Bethesda, Maryland 20892, USA, tel 001-301-496-4686; 001-301-4020574; stratakc@mail.nih.gov.

Conflict of Interest Statement

The authors have no conflict of interest to disclose

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improved the organization of cortical bone that was adjacent to the tumor. We conclude that, *in vitro* and *in vivo*, Celecoxib had an inhibitory effect on FD cell proliferation and in mouse FDLL structure, respectively. We speculate that COX-2 inhibitors offer an attractive alternative to current treatments for benign tumors such as OMX and FD that, apart from tumor suppression, may mechanically stabilize affected bones.

Keywords

cyclic AMP; prostaglandin E2; cyclooxygenase-2 (COX-2); bone tumors; McCune-Albright syndrome; Carney complex

Introduction

Carney complex (CNC), a genetic syndrome predisposing to multiple neoplasias (Stratakis et al., 2001 and Carney et al., 1985), is caused mostly by inactivating mutations of *PRKARIA*, a gene coding for the type 1a regulatory subunit (R1 α) of cAMP-dependent protein kinase (PKA) (Kirschner, Sandrini et al., 2000 and Kirschner, Carney et al., 2000). Mice with the *Prkar1a* gene entirely knocked out (KO) die early in embryonic development (Kirschner et al., 2009, Amieux McKnight, et al., 2002, and Amieux, Howe et al., 2002), whereas tissue-specific *Prkar1a* KOs in the adrenal cortex, heart, and bone developed various lesions that formed from resurgence or abnormal proliferation of stem-like cells (Yin et al., 2008, Sahut-Barnola et al., 2010, and Molyneux et al., 2010).

In the bone, cells of multipotent nature may be responsible for osteochondromyxoma (OMX), a benign neoplasm that is occasionally associated with primary pigmented nodular adrenal dysplasia (PPNAD) in the context of Carney complex (CNC) (Carney et al., 2001). In the *Prkar1a* heterozygote (*Prkar1a*^{+/-}) mouse (Kirschner et al., 2005) and in the mouse that was deficient in both *Prkar1a* and *Prkaca*, the main catalytic subunit of PKA (*Prkar1a*^{+/-} $\times$ *Prkaca*^{+/-}) (Tsang et al., 2010), abnormal proliferation of adult bone stromal cells (aBSCs) led to multiple tumors and fibrous dysplasia (FD)-like lesions (FDLL). Although previous studies have shown that *PRKARIA* or *GNAS* mutations share some downstream signals (such as activation of the MAPK pathway), they differ substantially on others (e.g. genes related to Wnt signaling) (Almeida et al., 2012). Nevertheless, in both these animal models aBSC neoplastic growth was fueled by prostaglandin E2 (PGE2) (Almeida et al., 2011). PGE2 is known to maintain stem cell-like cellular populations in various tissues (Greenhough et al., 2009, Daikoku et al., 2014 and Cha et al, 2006). Accordingly, Celecoxib, an approved-for-use in humans compound and effective inhibitor of the prostaglandin-endoperoxide synthase 2 (PTGS2) enzyme (also known as cyclooxygenase-2 or COX2) has been used with the intent to decrease neoplastic growth in a variety of tumors (Daikoku et al., 2014, Yokouchi et al., 2014, Kurtova et al., 2015 and Bertagnolli et al., 2006).

We hypothesized that Celecoxib might be effective in decreasing the proliferation of FD cells and treating FDLL and other bone tumors caused by *Prkar1a* haploinsufficiency: if COX2 and/or PGE2 have a role in maintaining and/or increasing the growth or proliferation of aBSCs (Almeida et al., 2011) in *Prkar1a*-haploinsufficiency (Kirschner et al., 2005, Tsang et al., 2010 and Pavel, et al., 2008), then their inhibition should be effective in the treatment

of these bone lesions. Our data support this hypothesis: both in vitro and in vivo, Celecoxib treatment resulted in decreased FD cell proliferation and inhibited, at least in part, the growth of these bone lesions. In addition, Celecoxib partly restored organization of cortical bone affected by the lesions.

Materials & Methods

Animal protocol

The *Prkar1a*^{+/-}/*Prkaca*^{+/-} (Tsang et al., 2010) mice have been described previously; they were maintained on a mixed genetic background (C57BL/6/129Sv). Mice were genotyped as described (Kirschner et al., 2005 and Tsang et al., 2010) and treated for 6 weeks with Celecoxib 1,500 ppm mixed in their NIH-31 diet (equivalent to 1,500 mg pre kg of diet) (n=16); control mice (n=16) with the same genotypes had access to the same NIH-31 diet without the medication. Mice between 6 and 12 months old were used based on the time that these animals developed bone lesions (Kirschner et al., 2005, Tsang et al., 2010 and Pavel et al., 2008).

All animal studies were performed under protocol ASP12-033 and were approved by and conducted in accordance with the *Eunice Kennedy Shriver* National Institute for Child Health and Human Development (NICHD) Institutional Animal Care and Use Committee. At necropsy, bone tissues were preserved for histopathologic and molecular studies. Formalin fixed, paraffin embedded sections were studied by immunostaining (see below).

Cell line and MTT cell proliferation assay

A human FD cell line (FD160) was established from biopsied materials produced from a patient with FD carrying the *GNAS1* mutation (R201C) (Boyce et al., 2012), propagated, and maintained as previously described (Bhattacharyya et al., 2012). The cell line was *PRKARIA* wild type (Reviewer 2, comment 1). Cell numbers were counted by Luna Automated Cell Counter (Logo Biosystems, Annandale, VA) after trypan blue staining.

The Vybrant MTT cell proliferation assay kit (Life Technologies, Grand Island, NY) was used to examine the effect of Celecoxib on cell growth rates. Cells were seeded at 5,000 cells per well in 96-well plate and cultured for 48 hours. Cells were treated with different concentrations of Celecoxib diluted in DMSO for 24 hours; cells without treatment were used as control and cells treated with DMSO were used as controls. Cell numbers after Celecoxib treatment were determined by MTT assay as a representative of cell growth rates. For each well, 10 µl MTT stock solution was added to 100 µl fresh medium and incubated at 37°C for 4 hours. 100 µl of SDS-HCl solution was then added to each well and incubated at 37°C for 16 hours in a humidified chamber. Absorbance was read at 570 nm.

FACS analysis of cell proliferation and cell cycle

FD160 cells with and without Celecoxib treatment were cultured for 48 hours. 10 µl/ml BrdU were added to both cultures and incubated for 60 minutes. Cells were then harvested, washed with PBS and resuspended in 100 µl PBS. 10ul Aqua viability dye (1:40) were added and incubated in room temperature for 10 minutes, followed by 1 ml of 1X FACS

Lysing Solution. Cells were then incubated with 300 µl Cytotfix/perm buffet at room temperature for 30 minutes, washed, pelleted and resuspended with 100 µl Perm Wash Buffer. 5 µl DNase was added and incubated at room temperature for 30 minutes, followed by 1 µl of BrdU-APC and 5 µl Ki67-FITC antibody and another 30 minutes incubation at room temperature. Fluorescent events were collected on a modified LSRII flow cytometer (BD Immunocytometry Systems, Franklin Lakes, NJ) and electronic compensation was performed with antibody capture beads (BD Biosciences, Franklin lakes, NJ) stained separately with antibodies used in the test samples. Data were analyzed using FlowJo Version 9.6 (TreeStar, Ashland, OR).

Haematoxylin/eosin (H&E) staining and other immunohistochemistry (IHC)

Tumors of the tail vertebrae were dissected from mice and fixed in 10% neutral buffered formalin for 24 hours, followed by decalcification in 10% formic acid (Decalcifier I, Surgipath Medical Industries Inc., Richmond, Illinois) for 24 hours and rinsing in running water for 30 mins. Fixed tissue was embedded into paraffin blocks, sectioned (5 µm) and H&E or IHC stained by Histoserv Inc (Germantown, MD). IHC staining was performed with mouse primary antibodies for ETS1, CASPASE1, IL1b and ki-67, (all from Santa Cruz Biotechnology, Inc., Dallas, TX).

Western blot analyses

Tissue lysates were extracted by T-PER tissue protein extraction reagent (Thermo Fisher Scientific, Rockford, IL), containing protease inhibitor cocktail I (EMD Biosciences, La Jolla, CA) with subsequent centrifugation at 10,000rpm for 10 minutes at 4°C. Protein concentration was determined by BCA protein assay (Thermo Fisher Scientific, Rockford, IL). Twenty micrograms of protein were mixed with 5 µL of 2x SDS protein gel loading solution and 5 µL deionized water, boiled for 5 min, and run on a Novex 10% Tris-glycine gel. Protein was transferred onto a 0.45 µm nitrocellulose membrane. The membrane was blocked with TBS containing 5% nonfat dried milk and 0.5% Tween 20. Proteins were detected with primary antibodies against the target proteins (CASPASE 1) and horseradish peroxidase-conjugated secondary antibodies against rabbit IgG (1:1,500). All antibodies were diluted in 5% nonfat dried milk in TBS-Tween 20. Bands were detected by enhanced chemiluminescence reagent.

In vivo double labeling of bone-forming surfaces with calcein injection

To evaluate growth rate of tumors in vivo, mice with visible vertebrae tumors were injected with calcein. Calcein solution was made by dissolving 3 mg calcein powder (Sigma-Aldrich, St.Louis, MO) in 1 ml 2% NaHCO₃ (0.2 g of NaHCO₃ in 10 ml double distilled H₂O, pH 7.4). 10 µg of calcein per gram of body weight was injected subcutaneously. The first injection was on day 1, the second injection was on day 9, and, sacrifice of the mice was on day 14.

Fluorescence and polarized microscopy of calcein labels and organization of cortical bone

Skinny tails were fixed overnight at room temperature in 0.7% paraformaldehyde (ThermoFisher, methanol-free from a freshly open ampule) dissolved in 0.5 mM CaCl₂, 0.7x

PBS. Vertebrae selected as described below were cryo-sectioned to 10 μm in coronal plane through the middle of the vertebrae. Sections with sharpest calcein lines in cortical bone were selected so that the section plane would be approximately perpendicular to the cortical bone surface. The sections were hydrated with PBS and sealed in glass slides with nail polish.

Bright-field transmission, polarized and fluorescence images of the cortical bone and adjacent soft tissues in the sections were recorded on the Olympus microscope with a 480nm band pass filter for fluorescence excitation. For each field of view, two polarized images were recorded at polarization angles of crossed polarizers set at 45 and 0 degrees with respect to mineralizing front of the cortex.

Rate of lateral tumor growth was evaluated via cortical bone growth rate. To make such evaluation possible, it was important to select vertebrae with osteomyxoma tumors that were at intermediate stages so that several criteria described in Results would be satisfied. Specifically, we selected proximal 3rd through 8th tail vertebrae which had visible tumors and whose width was smaller than 2/3 of the vertebrae length and which did not have any chondroma tissue detectable by microscopic observations. The cortical bone growth rate, also called mineral apposition rate in bone histomorphometry, is measured as the distance between the two calcein labels divided by the time interval between the two injections of calcein which labels bone forming surfaces. The rate was averaged over both cortical sides of a single section. Overall, about 3mm of cortical length per animal was analyzed, excluding the regions within 300 μm from the vertebrae ends. We disregarded small regions which were accidentally damaged during preparation and small (less than 2%) regions where calcein labels and osteocyte lacunas appeared wide due to bone forming surface being at a slanted angle with respect to the section plane, Figs. S1 and S2B. Some regions constituting about 10-30% of cortical length lacked the first label due to resorption of the cortical bone from the marrow cavity side. The growth rate for such regions was evaluated as the distance between the second label and the mineralizing front divided by the time interval between the second injection and the sacrifice. Position of the mineralizing front was determined from overlaid fluorescence and transmission images on which mineralized regions appear darker than non-mineralized ones. We verified that rates measured from the second label and the mineralizing front and from both labels were consistent.

Cortical bone disorganization was evaluated from the bright-field and polarized images. Normal cortical bone has three distinct features: (i) collagen fibers and (ii) osteocyte lacunae are laid parallel to the bone surface, and, (iii) osteocyte lacunae have regular shape. The affected cortical bone is heavily penetrated by Sharpey fibers running obliquely or perpendicularly to the bone surface (Tsang et al., 2010). The Sharpey fibers disrupted the parallel and organized deposition of collagen and osteocytes by osteoblasts, resulting in irregular appearance of collagen matrix and osteocytes in the polarized and bright-field images. The Sharpey fibers are thick and emanate from fibrous periosteal tissue covering bone surface. Once such fibers penetrate the cortex, their disappearance is expected to take substantial time, if the tumor effect on cortical bone formation is reversed. Therefore, we examined only the last 15 μm of bone matrix deposited before sacrifice (15 μm below the mineralizing front). We calculated fraction of cortex length with well-organized last layer

and averaged it over both cortical sides of a single section. Overall, about 3mm of cortical length per animal was analyzed, excluding the regions within 300 μ m from the vertebrae ends. Note that the Sharpey fibers perturbed but did not completely disrupt mineralizing front as indicated by wavy yet well-distinguishable calcein lines, Figs.S1 and S2.

TUNEL and EdU assays

For EdU assay, the mice were injected with EdU (100 μ g EdU in 100 μ l PBS) intraperitoneally 96 hours before sacrifice. Skinned tails were fixed overnight at room temperature in 0.7% paraformaldehyde from freshly open ampules dissolved in 0.5 mM CaCl_2 , 0.7x PBS. Proximal 3rd through 8th tail vertebrae whose width was smaller than 2/3 of the vertebrae length were selected and cryo-sectioned to 6 μ m in coronal plane through the middle of the vertebrae. Vertebrae with any detectable chondroma tissue were discarded. Alexa-488 EdU Kit (Life Technologies, Grand Island, NY) were used according to the manufacturers' instructions. Before mounting, the sections were washed in 5mM EDTA, 3% BSA, PBS solution for 30 minutes to remove the stains non-specifically bound to mineralized regions. The sections were mounted to DAPI-Fluoromount-G medium (EM Sciences, Hartfield, PA). Bright-field transmission and fluorescence images of the sections were recorded in an Olympus BX51 microscope. Similar Procedures were used for TUNEL assay, DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) were used following manufacture's instruction. Fraction of nuclei with overlapping DAPI and EdU or TUNEL label were counted. Osteocytes, muscles and ligaments were excluded from counting.

PGE2 measurements

A PGE2 ELISA Kit (abcam, Cambridge, MA) was used to measure serum PGE2 levels. 100 μ l of standard dilute, standards #1 to #7 and samples were pipetted into the appropriate wells, followed by 50 μ l blue conjugate and 50 μ l yellow antibody to standards and samples wells. Incubate at room temperature on a plate shaker for 2 hours at 500 rpm, and wash the plate 3 times. Add 200 μ l of pNPP substrate solution to each well and incubate at room temperature without shaking. Add 50 μ l of stop solution to every well and read the plate immediately. Read the optical density at 405 nm, preferably with correction between 570 and 590 nm. PGE2 levels in samples were calculated based on the standard curve generated from standards.

Quantitative real-time PCR (qPCR)

For each sample, 1 μ g of mRNA was reverse transcribed to cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in an Applied Biosystems 7500 real-time PCR system. mRNA expression levels of inflammasome pathway ligands were detected by caspase-1 (*Casp1*, Mm00438023_m1), interleukin 1 β (*Il1b*, Mm00434228_m1), PTGS2 (COX2) (*Ptgs2*, Mm00478374_m1). Gene expression levels were normalized to *Gapdh* (Taqman Rodent Gapdh control, Applied Biosystems, Foster City, CA) and control samples and expressed by DDCT as fold changes.

Statistical analysis

All analyses were performed using the two-tailed Student's t test. Differences at $p < 0.05$ were considered statistically significant. Mean $\pm$ SE of at least in-triplicate experiments were calculated.

Results

Responses of FD160 cell line to Celecoxib

Human cells isolated from a patient with FD (FD160) were used for this study (Boyce et al., 2012). The FD160 cell line with and without Celecoxib treatment was cultured for 48 hours. The FD160 cell culture without Celecoxib treatment reached confluency after 48 hours in culture (Figure 1A), while there was decreased cell density in the Celecoxib-treated culture (Figure 1B). When FD160 cells were treated with different concentrations of Celecoxib for 48 hours, MTT assay results showed a dose-dependent decline in cell counts as a result of Celecoxib (Figure 1C). We then studied the translational expression of caspase-1 by western blot analysis, and found decreased caspase-1 levels after Celecoxib treatment (Figure 1D). FACS analysis indicated that Celecoxib treatment also decreased proliferation (Figure 1E).

Celecoxib treatment of mice with bone lesions

After 6 weeks of Celecoxib treatment, partial rescue of the bone phenotype caused by *Prkar1a* and *Prkaca* loss (Kirschner et al., 2005 and Tsang et al., 2010) was observed. Pathology of the tail tumors from treated mice (n=16) showed lower density in the bone marrow space, with decreased fiber and bone formation, and reduced destruction of the adjacent cortical bone or growth plate (Figure 2A) compared with control mice (n=16). Immunohistochemical analysis also indicated decreased intensities of inflammasome pathway ligands and cell proliferation marker Ki67 after Celecoxib treatment (Figure 2B).

Growth of FDLL and bone tumors associated with *Prkar1a* and *Prkaca* haploinsufficiency was slow. Conventional tumor measurement methods (e.g. measuring size of each lesion) could not detect readily differences after Celecoxib treatment (data not shown). This is because errors of conventional methods are higher than tumor lateral size accrual during the 1.5 month treatment, with the accrual being estimated from Fig.3 to be approximately 180 μ m and 70 μ m, in untreated and treated mice, respectively. We adapted a method of mineral apposition rate measurement (Frost HM, 1983) to assess FDLL growth in our mice using injections of the fluorescing dye calcein which binds to bone-forming surfaces. Each of the two calcein injections identified the newly formed bone surface with green fluorescence, whereas the space in between reflected newly formed bone between the two calcein injections; within a fixed period of time, the larger the space between two calcein lines, the faster the new bone was forming, Figure 3A, B.

The idea of such tumor growth measurement via the cortical bone growth was based on the way FDLLs grow: After the initial displacement of the bone marrow in earlier phases, affected vertebrae expand in size. During this expansion and until late tumor stages, lesions remain "encapsulated" by cortical bone which is deposited on the external and removed from the internal side of the cortex. Under certain conditions, the mineralizing front of the

cortex follows lateral expansion of the tumor, and, therefore, rate of lateral expansion of the tumor should be equal to the mineral apposition rate of the cortex. Such conditions are listed below; they were ensured by selection of intermediate stage tumors as described in Methods and were verified in the Supporting Material: (1) During the assessed period of tumor growth, affected vertebrae remained encapsulated by cortical bone and gross morphology of the vertebrae was preserved. This was the case for the selected vertebrae, as evidenced by histology, Fig.1 A,B and S1. (2) Formation of mineralizing bone matrix outside the cortical bone, was negligible (less than 2%), Fig.S2A. (3) Cortical bone surface remained approximately parallel to the tumor surface, Figs. 3A, S1 and S2. (4) Cortical mineralizing fronts labeled by calcein were well defined in almost all (more than 97%) cortical length, Figs. 3A, S1 and S2. (5) Non-mineralized soft tissue between the cortical bone and the skin remained thin, Figs. S1 and S2. We verified further that the bone growth rate evaluated the tumor growth rate by measuring lateral tumor width in untreated mice from photographs of tails taken with 2.5 week intervals over long, 3.5 months period, Fig.S3.

We measured in such a way FDLL growth rate in treated and untreated *Prkar1a+/-Prkaca+/-* mice. In Celecoxib-treated mice, the tumor growth rate measured by calcein labeling was 2.5 times slower. We then assessed cortical bone organization in the intermediate stage tumors by bright-field and dark-field polarized microscopy. Bright-field images show order (shape and orientation) of lacunae in bone matrix where osteocytes reside; polarized images show the organization and direction of the collagen fibers in the extracellular matrix of bone. In affected vertebrae, normal cortical bone is resorbed and replaced by bone matrix which tends to have disorganized osteocytes and collagen fibers and is poorly mineralized and mechanically weak, as we have shown before (Tsang et al., 2010). During the slowed down growth of cortical bone in the treated mice, the fraction of well-organized cortical bone increased 3-fold, as we measured from these polarized and bright field images (Figure 4). Celecoxib treatment also decreased proliferation (Figure 5A) and increased apoptosis, as measured by TUNEL (Figure 5B), within mouse FDLLs and other bone lesions.

Changes in PGE2 levels and gene expression after Celecoxib treatment

As expected, Celecoxib treatment reduced PGE2 levels in *Prkar1a+/-Prkaca+/-* vertebral bone, as indicated by PGE2 ELISA (Figure 6A). The qPCR analysis showed significant effects Celecoxib treatment on bone, inflammatory, and other gene expression. We found significantly decreased expression of *interleukin-1b (Il1b)*, *caspase-1 (Casp1)*, *Ets-1* and *COX2 (Ptg2)* in the vertebral bone tumors of Celecoxib-treated mice (Figure 6B) compared to controls with a regular NIH-31 diet.

Discussion

Celecoxib, uniquely among all non-steroid anti-inflammatory drugs (NSAIDs), has been used extensively both in vitro and in vivo to inhibit neoplastic growth or proliferation (Greenhough et al., 2009, Daikoku et al., 2014, Yokouchi et al., 2014, Kurtova et al., 2015 and Bertagnolli et al., 2006). Some of the in vitro effects may be because at certain concentrations the drug is insoluble and causes precipitate-dependent effects and cellular toxicity (Sacchetti, A., 2013). However, there are also true biologic effects of Celecoxib,

mostly linked to inhibition of PGE2 synthesis and the consequent decrease of this molecule's induction of stem cell-like cells that are essential for the growth of at least some tumors (Kurtova et al., 2015, Tsujii et al., 1998 and Mazhar et al., 2006).

We hypothesized that Celecoxib might be useful in the treatment of FDLL, because both in animal models (Kirschner et al., 2005, Tsang et al., 2010, Pavel et al., 2008, Wu et al., 2011, Chagin et al., 2014 and Bianco et al., 1998) and in humans with CNC and McCune Albright syndrome (Carney et al., 2001, Tsang et al., 2010, Boyce et al., 2012, Bhattacharyya et al., 2012 and Zhang et al., 2012), FD and related tumors have been linked to proliferation of aBSCs and related stem-like cells of the bone. In addition, in mice with OMX and FDLL due to *Prkar1a* deficiency, PGE2 levels were elevated both at the tumor level and systemically, and linked to abnormal growth and proliferation of aBSCs (Almeida et al., 2011). Indeed, our data support an effect of systemic Celecoxib therapy in both human FD cells and mouse FDLL caused by *Prkar1a* deficiency.

COX2, and its product, PGE2, have a number of effects on bone, as a number of previous studies have demonstrated (Almeida et al., 2011, Zhang et al., 2012, Zhang et al., 2002, Shim et al., 2010, Blackwell et al., 2010). More than four decades ago, PGE2 was shown to stimulate cAMP in bone cultures (Klein and Raisz, 1970). Both FD caused by *GNAS1* mutations and CNC caused by downstream PKA defects may be connected by increased cAMP signaling via activation of PGE2 receptors present on bone cells. Since, it has become clear that almost all aspects of bone formation, including resorption, turnover, healing and aging may be affected by COX2 and PGE2 (Zhang et al., 2012). In addition, COX-2 is expressed in cells of the hematopoietic lineage that are an essential part of the bone microenvironment (North et al., 2007). Patients with mutations of enzyme 15-hydroxyprostaglandin dehydrogenase (PDGH) that degrades PGE2 develop primary idiopathic hypertrophic osteoarthropathy (Uppal et al., 2008) and bone repair and healing are impaired by COX2 inhibitors, pointing to the osteogenic capacity of PGE2 (Simon and O'Connor, 2007 and Alander and Raisz, 2006). Accordingly, in men, COX-2 inhibitor use has been associated with lower hip and spine bone mineral density (Richards et al., 2006). In addition, there is clinical data indicating that Celecoxib inhibits heterotopic ossification postoperatively, after total hip arthroplasty (Oni et al., 2014 and Lavernia et al., 2014).

The present study suggests that Celecoxib treatment led to decreased proliferation of FD cells. Although not proven in the present study, we speculate that the decreased proliferation and increased apoptosis of FDLL tumor cells treated with Celecoxib were due to an effect on the primary cells responsible for the phenotype. These are aBSCs with *Prkar1a* haploinsufficiency, whose continued proliferation in mouse skeleton causes tumors (Kirschner et al., 2005, Tsang et al., 2010 and Pavel et al., 2008). PGE2 is essential for maintaining stem cell-like cells in a number of tissues (Greenhough et al., 2009, Daikoku et al., 2014 and Cha et al., 2006) and inhibition of the enzyme that degrades PGE2 (PDGH) enhances regenerative capacity (Zhang et al., 2015). We also showed that treatment with celecoxib caused a significant decrease in the expression of inflammasome-related genes. We were interested in this particular pathway, given that in a previous study by our group (Almeida et al. 2011) had shown a link between abnormal cAMP signaling and defective regulation of the inflammasome in bone lesions of neonatal-onset multisystem inflammatory

disease (NOMID) patients. These NOMID lesions are very similar to the bone lesions of this study.

More importantly, the treatment improved the histologic phenotype and cortical bone organization of FDLL associated with *Prkar1a* deficiency, although a weakness of the study is that we did not study markers of bone formation and turnover and focused only on structural changes. We hypothesized that this improvement may be due to an effect of Celecoxib on tumor cells which are present in the periosteum and result in formation of Sharpey fibers which “pierce” and disorganize the cortical bone like Sharpey fibers do in FD lesions (Bianco et al., 1998). Because the affected cortical bone is disorganized and mechanically weak, the improvement of cortical bone organization may improve mechanical integrity of the whole bones with lesions and delay amputation of affected bones.

Our study had limitations in addition to not studying markers of bone turnover and formation: although there might be similarities between human FD and FDLL in mice, and CNC and FD do arise from changes in the same signaling pathway, one could argue that the use of human FD cells and mouse FDLL lesions to test a potential treatment does not test the same pathophysiology. Our group has shown, however, the similarities between skeletal lesion from *prkar1a/prkarca* double heterozygous mice and human FD. In addition, the dual calcein labeling is an indirect method for tumor growth measurement that we had to use due to slow tumor growth and insufficient accuracy of traditional methods. However, we verified the underlying assumptions and validated this method, as mentioned in our results section. Although our data support a possible role of Celecoxib in the treatment of patients with FD or CNC, human studies have not been performed. Prior experience has shown a consistent benefit from celecoxib in prevention of colonic adenoma recurrence (Arber et al. 2006) rather than in the treatment of colorectal cancer (Kohne et al. 2007). Given the potential adverse effects (gastroduodenal toxicity and increased cardiovascular risk) (Essex et al., 2013) any benefits of long-term celecoxib therapy must be weighed against this risk. Future human studies have to be planned carefully and include a number of dosing regimens in the best possible setting.

In conclusion, this pilot study of Celecoxib, a COX2 inhibitor, showed promising results in the treatment of mice with bone lesions caused by *Prkar1a* deficiency. More studies are needed but it is possible that Celecoxib and other NSAIDs that decrease PGE2 levels provide a useful addition to the agents currently being used for the treatment of FD and related bone neoplasms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements/Funding

This work was supported by the Intramural Research Program (IRP) of the *Eunice Kennedy Shriver* National Institute of Child Health & Human Development (NICHD), National Institutes of Health (NIH), Bethesda, MD 20892 USA and a grant by the Hellenic Society of Medical Oncology (to Dr. Saloustros).

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Highlights

- Effect of systemic Celecoxib therapy in both human FD cells and mouse FDLL caused by *Prkar1a* deficiency
- *In vitro* and *in vivo* Celecoxib treatment led to decreased proliferation of FD cells and FDLLs.
- Celecoxib treatment in mice improved the histologic phenotype and cortical bone organization of FDLLs associated with *Prkar1a* deficiency
- This study presents promising results for the treatment of mice with bone lesions caused by *Prkar1a* deficiency.
- POSSible role of Celecoxib in the treatment of patients with FD or CNC; however, to date no such human trail has been conducted.
- It is indeed possible that Celecoxib (and other NSAIDs that decrease PGE2 production) provide a useful addition to the agents currently being used for the treatment of FD and related bone neoplasms in humans.

Figure 1.

Effect of Celecoxib treatment on human cell line FD160. **A.** Image of control FD160 cells; cells reached confluence after 48 hours in culture. **B.** Image of Celecoxib treated FD160 cells; 48 hours of Celecoxib treatment led to a lower cell density in culture **C.** Cell viability studied by the MTT cell proliferation assay: decreased cell viability was seen with increasing Celecoxib concentrations. **D.** Western blot analysis. Celecoxib treatment led to decreased caspase-1 expression on the protein level. **E.** FACS analysis of the effect of Celecoxib on FD160 cell proliferation.

Figure 2. Effect of 6 weeks Celecoxib treatment on vertebral bone lesions of *Prkar1a+/-Prkaca+/-* mice. Representative images from 4 mice and 12 lesions in total (3 per animal) are shown. **A.** Celecoxib treatment partially rescued the fibrous lesions inside vertebral bone marrow. **B.** Immunohistochemical staining showed decreased staining for inflammasome pathway ligands ETS1, Caspase-1 and IL-1B, and cell proliferation marker Ki67.

Figure 3.

Effect of celecoxib treatment on tumor growth rate measured by *in vivo* calcein double labeling and validation of the measurement. (**A**) Overlaid bright-field (grey) and fluorescence (green) images of calcein labels of the forming surfaces of cortical bone encapsulating the tumor. (**B**) Tumor growth rate measured by the labeling. The radial growth rate is the mineral apposition rate averaged over two cortical sides of a vertebra. Symbols are for individual animals, columns are averages over all animals, and, error bars are standard errors of the mean.

Figure 4.

Effect of celecoxib treatment on cortical bone organization in affected tail vertebrae. (**A**) Bright-field and polarization microscopic images of a mid-coronal section of a vertebra. Mineralized cortex appears darker on the bright-field image and is on the left of the mineralizing front marked by arrows. The disorganized cortical region appears darker and less uniform in the polarized image. (**B**) Fraction of length of well-organized cortex. Bars are standard errors of the mean.

Figure 5.

Effect of celecoxib treatment on proliferation and apoptosis in affected tail vertebrae. **(A)** Overlaid DAPI and EdU-Alexa-594 fluorescence images of a mid-coronal section of a vertebra. Arrowheads mark nuclei stained with EdU-Alexa-594. Diffuse or extended red staining is due to non-specific adsorption of Alexa-594 to mineralized regions. **(B)** DAPI and TUNEL-fluorescein fluorescence images. Arrowheads mark apoptotic nuclei stained with DAPI and TUNEL-fluorescein. Bars are standard errors of the mean.

Figure 6. Celecoxib treatment affect expression of inflammasome and wnt pathway ligands. **A.** PGE2 ELISA was used to study changes in PGE2 levels after Celecoxib treatment. **B.** RT-PCR study indicated decreased transcriptional expression of inflammasome pathway.