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Genetic background influences fluoride's effects on osteoclastogenesis

Dong Yan [a], Aruna Gurumurthy [a], Maggie Wright [c,d], T. Wayne Pfeiler [c,d], Elizabeth G. Loboa [c,d], Eric T. Everett [b,*]

[a] *Dental Research, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*
[b] *Department of Pediatric Dentistry, School of Dentistry and The Carolina Center for Genome Sciences, 3140 Old Dental Building, CB #7450, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7450, USA*
[c] *Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*
[d] *North Carolina State University, Raleigh, NC, USA*

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Abstract

Excessive fluoride (F) can lead to abnormal bone biology. Numerous studies have focused on the anabolic action of F yet little is known regarding any action on osteoclastogenesis. Little is known regarding the influence of an individual's genetic background on the responses of bone cells to F. Four-week old C57BL/6J (B6) and C3H/HeJ (C3H) female mice were treated with NaF in the drinking water (0 ppm, 50 ppm and 100 ppm F ion) for 3 weeks. Bone marrow cells were harvested for osteoclastogenesis and hematopoietic colony-forming cell assays. Sera were analyzed for biochemical and bone markers. Femurs, tibiae, and lumbar vertebrae were subjected to microCT analysis. Tibiae and femurs were subjected to histology and biomechanical testing, respectively. The results demonstrated new actions of F on osteoclastogenesis and hematopoietic cell differentiation. Strain-specific responses were observed. The anabolic action of F was favored in B6 mice exhibiting dose-dependent increases in serum ALP activity ($p<0.001$); in proximal tibia trabecular and vertebral BMD (tibia at 50&100 ppm, $p=0.001$; vertebrae at 50 and 100 ppm, $p=0.023$&0.019, respectively); and decrease in intact PTH and sRANKL ($p=0.045$ and $p<0.001$, respectively). F treatment in B6 mice also resulted in increased numbers of CFU-GEMM colonies ($p=0.025$). Strain-specific accumulations in bone [F] were observed. For C3H mice, dose-dependent increases were observed in osteoclast potential ($p<0.001$), *in situ* trabecular osteoclast number ($p=0.007$), hematopoietic colony forming units (CFU-GEMM: $p<0.001$, CFU-GM: $p=0.006$, CFU-M: $p<0.001$), and serum markers for osteoclastogenesis (intact PTH: $p=0.004$, RANKL: $p=0.022$, TRAP5b: $p<0.001$). A concordant decrease in serum OPG ($p=0.005$) was also observed. Fluoride treatment had no significant effects on bone morphology, BMD, and serum PYD cross-links in C3H suggesting a lack of significant bone resorption. Mechanical properties were also unaltered in C3H. In conclusion, short term F treatment at physiological levels has strain-specific effects in mice. The expected anabolic effects were observed in B6 and novel actions hallmarked by enhanced osteoclastogenesis shifts in hematopoietic cell differentiation in the C3H strain.

Keywords: F; Osteoclastogenesis; Hematopoietic colony-forming cell (CFC) assays; Bone resorption markers; Inbred mouse strains

Introduction

Fluoride (F) is an important micronutrient that accumulates within mineralized tissues such as teeth and bone [1,2]. In addition to dental/enamel fluorosis, excessive systemic F can lead to skeletal fluorosis a condition hallmarked by osteosclerosis, ligament calcifications, and often accompanying osteoporosis, osteomalacia, or osteopenia [3,4]. F's known actions on bone can be mediated through direct physicochemical interactions [5,6] and as an anabolic agent capable of affecting osteoblasts *in vitro* [2] and *in vivo* [7]. As an anabolic agent, F is capable of increasing bone mass through an undetermined mechanism on osteoblasts [8]. Due to the anabolic action of F, its potential use as an agent for the treatment of postmenopausal osteoporosis was explored with mixed results [9,10]. While NaF may increase bone mass, the new bone lacks normal structure and strength [11,12]. These observations in humans have been extended in rodents [13,14]. The role of

* Corresponding author. Fax: +1 919 966 3683.
E-mail addresses: dong_yan@dentistry.unc.edu (D. Yan), aruna_gurumurthy@dentistry.unc.edu (A. Gurumurthy), mkwright@unity.ncsu.edu (M. Wright), wayne_pfeiler@ncsu.edu (T.W. Pfeiler), egloboa@ncsu.edu (E.G. Loboa), eric_everett@dentistry.unc.edu (E.T. Everett).

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genetics/genetic background in F responses has been demonstrated in dental fluorosis [15,16]. In order to investigate more deeply F's effects on bone and bone cells, we chose to utilize C57BL/6J (B6) and C3H/HeJ (C3H) mice that have been extensively characterized for their bone and bone cell properties [17–20]. These two genetically distinct inbred strains of mice are commonly known as B6 with low bone mass and C3H with high bone mass. Compared with B6 mice, C3H mice have higher peak bone density [21,22], lower rate of bone resorption [17,23], and higher serum alkaline phosphatase (ALP) activity [23]. The current study was undertaken to test the hypotheses that F responsive variations in bone metabolism are different between B6 and C3H mice; to assess bone resorption by the osteoclasts that developed *in vitro* in the difference.

Materials and methods

Animals

Female B6 and C3H inbred mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) at 3 weeks of age and were acclimated for 1 week prior to treatment with NaF. NaF was provided in the drinking water at concentrations of 0 ppm, 50 ppm, and 100 ppm F ion for 3 weeks. Each treatment/control group consisted of 6 mice. All animals were housed in the Division of Lab Animal Medicine facility within the Dental Research Center, a fully AAALAC accredited unit, and were maintained on a 12:12 h light/dark cycle with an ambient temperature of 21 °C. Mice were fed a constant nutrition LabDiet® 5001 (PMI® Nutrition International), which contained 0.95% calcium, 0.67% phosphorous, 4.5 IU/gm vitamin D3, and an average [F] of 6.56±0.28 μg/gm. All experimental procedures were approved by the Institutional Animal Care and Use Committee at The University of North Carolina at Chapel Hill.

Sample collecting

Serum was collected from each mouse and then frozen at −80 °C until used. Femurs, tibiae, and lumbar vertebrae from each mouse were dissected free of soft tissues. The right femurs were stored at −20 °C prior to μCT and biomechanical testing. The tibiae were fixed in ice cold 10% neutral buffered formalin (10%NBF), analyzed by μCT, decalcified with buffered 0.25 M EDTA (pH 7.5) for 1 week, and processed for standard paraffin embedding and sectioning. Lumbar vertebrae were stored at −20 °C prior to μCT. Bone marrow cells were flushed from the left femurs and left tibiae with α-MEM and the erythrocytes lysed with 0.8% ammonium chloride.

Bone fluoride content

Femur samples were cleaned by removed the soft tissues, split open, and cleaned of all marrow with cold PBS. The specimens were dried at room temperature for 30 min, and then were defatted for 24 h treatment of chloroform, another 24 h treatment of ethyl ether with three changes, respectively. Prior to ashing, the dried/defatted bones were weighed. The bones were ashed at 600 °C for 8 h and weighed again, dissolved in 1 N HCl, and neutralized by 1 N NaOH, and F concentration in each specimen was measured by the ion-specific F electrode, Orion 720A+ advanced ISE/pH/mV/ORP meter (Thermo Electron Corporation, USA, 2003).

Serum ELISA/EIA and chemistry assays

Sera from each animal were subjected to several immunoassays that assessed surrogate bone metabolism markers including intact parathyroid hormone (PTH) (Mouse) EIA (ALPCO Diagnostics, Salem NH); soluble receptor activator of nuclear factor (NF)-κB ligand (sRANKL, the main stimulatory factor for the formation of mature osteoclasts and essential for their survival); mouse/rat EIA (ALPCO Diagnostics, Salem NH); mouse/rat osteoprotegerin (OPG, also called osteoclast inhibitory factor, a decoy receptor for RANKL); EIA (ALPCO Diagnostics, Salem NH); osteoclast-derived tartrate-resistant acid phosphatase form 5b (TRAP5b, a useful marker of bone resorption rate); MouseTRAP™ assay EIA (Immunodiagnostic Systems, Inc., Fountain Hills, AZ and SBA Sciences, Turku, Finland); mouse osteocalcin EIA (Biomedical Technologies, Inc., Stoughton, MA); and serum pyridinoline cross-link (PYD) (Metra Serum PYD, Quidel Corp., San Diego, CA). Each assay included adequate controls with known levels of target markers for quality control. The intra- and inter-assay precision (coefficient of variation, %) for these EIA/ELISA assays were 3.2% and 8.4% (PTH intra- and interassay, respectively); 4.2% and 9.0% (sRANKL intra- and inter-assay, respectively); 7.5% and 9.0% (OPG intra- and inter-assay, respectively); <6.5% and <8.0% (TRAP5b intra- and inter-assay, respectively), 6% and 8% (osteocalcin intra- and inter-assay, respectively), and 6–15% and 9–12% (PYD intra- and inter-assay, respectively). Sera from each animal were subjected to a suite of serum clinical chemistry tests performed that included albumin, total alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), BUN, creatinine, Na, K, Cl, total Ca, PO_4, Mg, and F. Microdirect analysis of serum F was performed according to the method of Vogel et al. [24].

In situ bone osteoclast numbers

Quantitation of osteoclasts in demineralized bone sections was based on previously described procedures [17,25]. Fixed, demineralized, and paraffin embedded tibiae were sectioned and subjected to staining for tartrate resistant acid phosphatase (TRAP) with 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml fast red violet LB salt in 0.1 M sodium acetate buffer, pH 5.0, containing 50 mM sodium tartrate [26]. Osteoclasts were identified as TRAP positive cells on the trabecular bone surfaces. The numbers of osteoclasts on the sections were counted and the fractions of bone surface occupied by osteoclasts were measured in the proximal tibia trabeculae using a color camera microscopy imaging system (Nikon ECLIPSE 50i and Nikon Digital Camera DXM1200F, Japan) and the software of ImageJ (1.35s, NIH).

Osteoclast potential

The bone marrow cells were cultured (1.0×10^5 cells/0.5 ml per well in a 48-well plate) for 6 days in α-MEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were fed every 3 days with medium containing rmM-CSF (20 ng/ml) (R&D, Minneapolis, MN) and rhsRANKL (60 ng/ml) (PeproTech Inc., Rocky Hill, NJ) and maintained at 37 °C in a humidified atmosphere of 5% CO_2. On day 6, cells were fixed in 10% formalin and stained for TRAP with 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml fast red violet LB salt in 0.1 M sodium acetate buffer, pH 5.0, containing 50 mM sodium tartrate [26]. TRAP-positive cells with three or more nuclei were counted using phase-contrast microscopy (Nikon ECLIPSE TS100, Japan).

Hematopoietic colony forming cell (CFC) assays

Hematopoietic colonies were obtained by growing bone marrow cells in Methocult GF+ media (StemCell Technologies, Vancouver) consisting of 1% methylcellulose in Iscove's MDM, 15% Fetal Bovine Serum, 1% Bovine Serum Albumin, 10 μg/ml rh Insulin, 200 μg/ml Human Transferrin (Iron saturated), 10^{-4} mM 2-Mercaptoethanol, 2 mM L-glutamine, 50 ng/ml rm Stem Cell Factor, 10 ng/ml rm IL-3, 10 ng/ml rh IL-6, and 3 units/ml rh Erythropoietin. The bone marrow cell populations were aliquoted at 2×10^4 per 35 mm dish and cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. After 14 days, the dishes were scored for colony-forming units (CFUs) according to standard criteria. In some cases, colonies were plucked and subjected to cytospin and stained with Giemsa to confirm cellular composition. *In situ* staining of methylcellulose colonies with benzidine was used to identify colonies containing erythroid cells. The numbers of different colonies (CFU-GEMM: Colony-Forming Unit-Granulocyte, Erythrocyte, Macrophage, and Megakaryocyte. CFU-GM: Colony-Forming Unit-Granulocyte, Macrophage. CFU-G: Colony-Forming Unit-Granulocyte. CFU-M: Colony-Forming Unit-Macrophage. BFU-E: Burst-Forming Unit-Erythrocyte) were counted in each dish using phase-contrast microscopy (Nikon ECLIPSE TS100, Japan).