CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

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Fluoride's Effects on the Formation of Teeth and Bones, and the Influence of Genetics

ABSTRACT

Fluorides are present in the environment. Excessive systemic exposure to fluorides can lead to disturbances of bone homeostasis (skeletal fluorosis) and enamel development (dental/enamel fluorosis). The severity of dental fluorosis is also dependent upon fluoride dose and the timing and duration of fluoride exposure. Fluoride's actions on bone cells predominate as anabolic effects both *in vitro* and *in vivo*. More recently, fluoride has been shown to induce osteoclastogenesis in mice. Fluorides appear to mediate their actions through the MAPK signaling pathway and can lead to changes in gene expression, cell stress, and cell death. Different strains of inbred mice demonstrate differential physiological responses to ingested fluoride. Genetic studies in mice are capable of identifying and characterizing fluoride-responsive genetic variations. Ultimately, this can lead to the identification of at-risk human populations who are susceptible to the unwanted or potentially adverse effects of fluoride action and to the elucidation of fundamental mechanisms by which fluoride affects biomineralization.

KEY WORDS: bone, enamel, fluoride, fluorosis, mice.

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INTRODUCTION

Fluorine is a common element in the earth's crust. Fluorides are naturally present in the soil, rocks, and water throughout the world, with higher concentrations in areas where there have been recent/past pyroclastic activities or geologic uplift. Fluorides are also widely used in many industrial processes. The major sources of systemic fluoride exposure are the diet (food and water) (USDA National Fluoride Database of Selected Beverages and Foods – 2004, http://www.nal.usda.gov/fnic/foodcomp/Data/Fluoride/Fluoride.html) and fluoride-containing dental products.

Fluoridation of community drinking water to prevent dental caries is considered as one of the ten most important public health achievements of the 20th century (Achievements in Public Health, 1900-1999: Fluoridation of Drinking Water to Prevent Dental Caries, 1999). Concurrent with the decline in dental caries has been an increase in the prevalence of dental fluorosis, a side-effect of fluoride exposure. Dental fluorosis remains highly prevalent world-wide. As recently as 2005, 23% of persons in the United States aged 6 to 39 years had very mild or greater enamel fluorosis (Beltran-Aguilar *et al*., 2005).

Historically, biochemists and protein chemists have used fluoride in millimolar (mM) concentrations as an enzyme inhibitor (*e.g*., phenylmethylsulfonyl fluoride is an inhibitor of a variety of serine proteases, whereas sodium fluoride is used as a general inhibitor of protein phosphoseryl and phosphothreonyl phosphatases). Efforts to understand additional actions of fluoride in cells, tissues, and organisms have been hampered by the use of a wide range of fluoride exposures, different fluoride salts (AlF_n, BeF_n, or NaF), or organic moieties of fluorine. Studies involving humans have focused on fluoride alone or in combination with other molecules (*i.e*., calcium). Studies involving animals vary in fluoride-dosing schedules as well as routes of administration (orally or parenterally). Finally, fluoride can demonstrate biphasic actions (*i.e*., mitogenic at low concentrations and toxic at higher concentrations). Collectively, this has complicated our understanding of the physiological effects of fluoride. Within this decade, the interaction of an individual's genetic background has offered new insight into fluoride's physiological effects. This review will focus on separate but often overlapping areas of fluoride action and genetic influences in modulating an animal's response to fluoride.

CELL SIGNALING, PROLIFERATION, STRESS, AND APOPTOSIS

As alluded to above, fluoride is part of the environment, whether one considers systemic exposure in whole animals or as part of the tissue culture milieu. Mitogen-activated protein kinases (MAPKs) signaling pathways allow cells to respond to myriad extracellular stimuli. The Jun N-terminal kinase (JNK) and p38 MAPK pathways are often activated following environmental stress (Weston and Davis, 2007; Wagner and Nebreda, 2009). Activation of these pathways can

lead to diverse responses, including cell proliferation, differentiation, survival, and apoptosis. Sodium fluoride appears to act predominantly through the Jun N-terminal kinase (JNK) and p38 MAPK signaling pathways (Thrane *et al*., 2001; Y Zhang *et al*., 2007; Karube *et al*., 2009). An alternative MAPK / ERK1 signaling pathway involving an osteoblastic fluoride-sensitive phosphotyrosyl phosphatase (PTP) has been proposed in regard to the mitogenic effects of fluoride (Thomas *et al*., 1996; Wu *et al*., 1997; Lau and Baylink, 1998). When fluoride is associated with metals like aluminum and beryllium, more complex signaling pathways appear to be evoked (Lau *et al*., 1991, 2002; Caverzasio *et al*., 1996, 1997; Susa, 1999; Misra *et al*., 2002; Li, 2003). Finally, in ameloblasts there is evidence that the Rho/ROCK pathway is activated by modest fluoride exposure (Li *et al*., 2005).

Fluoride can have deleterious effects on cells, depending upon concentration (micromolar to millimolar), duration of exposure, and cell type (primary cultured cells or established cell lines). Exposures up to 1 mM of NaF failed to induce stress-response RNAs or initiate apoptosis in the mouse odontoblast cell line M06-G3 (Wurtz *et al*., 2008), whereas 1 mM NaF induced oxidative stress and apoptosis in rat primary hippocampal neurons (M Zhang *et al*., 2007). Exposures in the 5- to 10-mM range are required to induce apoptosis in rat thymocytes and human gingival fibroblasts, rat primary lung cells, and in the odontoblast cell line MDPC-23 (Thrane *et al*., 2001; Matsui *et al*., 2007; Lee *et al*., 2008; Karube *et al*., 2009), and micromolar NaF leads to apoptosis in neonatal rat osteoblasts and fetal human ameloblast lineage cells (Q Yan *et al*., 2007; X Yan *et al*., 2009). Rats exposed to 0, 10, 50, or 100 ppm F ions (approximately 0 to 5.0 mM F ions) in the drinking water over a period of 10 wks demonstrated increases in reactive oxygen species in the blood at 50 ppm and 100 ppm, without evidence of significant oxidative stress within the brain or liver (Chouhan and Flora, 2008). In the kidney, there was increased oxidative stress only in the 100-ppm-F group. Analysis of these data together suggests tissue-specific sensitivity to oxidative stress following fluoride exposure.

Endoplasmic reticulum (ER) stress may be a component of fluoride's effects on amelogenesis. Depending upon the chemical form and concentration, fluoride can induce ER stress (Thrane *et al*., 2001; Misra *et al*., 2002; Kubota *et al*., 2005; Sharma *et al*., 2008; Ito *et al*., 2009). ER stress leading to protein misfolding can result from several insults. To alleviate the accumulation of unfolded proteins in the ER lumen, the unfolded protein response (UFP) is initiated. Two primary mechanisms are in place to deal with the accumulation of unfolded/misfolded proteins: increased transcription of genes encoding the protein chaperones and folding catalysts to up-regulate the cell's protein-folding capacity; and decreased biosynthetic burden of the secretory pathway in the cell by down-regulating expression of genes encoding secreted proteins (Rutkowski and Kaufman, 2004; Shen *et al*., 2004; Schroder and Kaufman, 2005). The reduction of incoming proteins appears to be mediated at the level of translation. Additionally, the UFP response leads to transcriptional up-regulation for adaptation or for apoptosis (Kaufman, 1999; Malhotra and Kaufman, 2007). Within the ER stress cascade is the activation of c-Jun amino-terminal kinases (JNKs) *via* mammalian homologs of yeast IRE1, which activate chaperone genes in response to ER stress (Urano *et al*., 2000). As secretory cells, ameloblasts may be particularly sensitive to induction of ER stress. Recently, the predominant secreted protein amelogenin has been found to bind to calnexin (a type I integral ER membrane chaperone that facilitates protein folding) in a yeast two-hybrid assay (Wang *et al*., 2005).

ACTIONS ON BONE AND BONE CELLS

Fluoride's actions on bone appear to be mediated at several levels. Fluoride can directly interact with the bone mineral matrix physicochemically (Grynpas, 1990; Grynpas and Rey, 1992; Pak *et al*., 1995; Chachra *et al*., 1999). *In vitro* fluoridation of bone with mM [F] can lead to conversion of carbonated hydroxylapatite to carbonated fluorapatite, to changes in crystallinity, and to a reduction in mechanical strength properties (Silva and Ulrich, 2000; Freeman *et al*., 2001; DePaula *et al*., 2002). Fluorapatite is more stable and resistant to acid dissolution than is hydroxyapatite (Grynpas and Cheng, 1988). Fluoride also delays mineralization and is capable of altering bone crystal structure (Grynpas, 1990; Grynpas and Rey, 1992; Mousny *et al*., 2008).

In addition to physicochemical actions, fluoride at 10^{-5} M and 10^{-7} M can influence matrix metalloproteinases (MMPs), affecting the composition of the remodeling matrix and subsequent mineralization in a rat *in vitro* mineralizing bone cell culture (Waddington and Langley, 2003).

Fluoride can act on osteoblasts and osteoclasts *in vivo* and *in vitro*. NaF is an anabolic agent capable of increasing bone mass, although the mechanism for its action remains unclear (Farley *et al*., 1983; Hall, 1987; Aaron *et al*., 1991; Lau and Baylink, 1998). While NaF may increase bone mass, the newly formed bone appears to lack normal structure and strength (Carter and Beaupre, 1990; Riggs *et al*., 1990; Søgaard *et al*., 1994). In trabecular bone, fluoride results in an increase in bone volume and trabecular thickness without a concomitant increase in trabecular connectivity (Aaron *et al*., 1991). It is this lack of trabecular connectivity that reduces bone quality despite the increase in bone mass. These observations in humans have been extended in rodents (Søgaard *et al*., 1995; Turner *et al*., 1995). At low local concentrations such as those that occur following fluoride modification of dental implants, there is enhanced osteoblastic differentiation and interfacial bone formation concomitant with increased expression of osteogenic markers at the implant site (Cooper *et al*., 2006; Monjo *et al*., 2008). When fluoride-substituted apatite is implanted *in vivo,* there is an increase in new bone formation when the percentage of fluoride is low (0.5% by weight); however, when the percentage fluoride is higher (2.23% by weight), the enhanced new bone formation is abrogated (Inoue *et al*., 2005). High systemic fluoride exposures can lead to skeletal fluorosis, a condition hallmarked by osteosclerosis, ligament calcifications, and often accompanying osteoporosis, osteomalacia, or osteopenia (Christie, 1980; Wang *et al*., 2007). Skeletal fluorosis can be complicated by malnutrition (Teotia and Teotia, 2008).

Fluoride can inhibit the function of isolated osteoclasts at modest [F] 0.5- to 1.0-mM (Okuda *et al*., 1990) or near-physiologic [F] 15-mg/L (approximately 0.8 mM) concentrations (Taylor *et al*., 1989) when examined *in vitro*. At a range of NaF from 0.15 mg/L to 30 mg/L (approximately 0.004 mM to 0.7 mM), biphasic effects of fluoride have been described, depending upon concentration in the culture media (Taylor *et al*., 1990). At 15-30 mg/L, osteoclast-resorptive activity was inhibited. However, at 1 mg/L (approximately 0.050 mM), osteoclast function was enhanced. The authors were unable to determine if the enhanced osteoclast function at 1 mg/L was a direct effect or indirect *via* an action on residual osteoblasts present in the culture system. Systemic fluoride treatment (50 ppm F ion in the drinking water over a period of 3 wks) results in the induction of osteoclastogenesis in the C3H/HeJ (C3H) inbred mouse strain, as evidenced by an increased number of osteoclasts along the trabecular bone surface in the tibiae (D Yan *et al*., 2007).

ACTIONS ON AMELOBLASTS

Dental fluorosis (DF) is an undesirable developmental defect of tooth enamel attributed to greater-than-optimal systemic fluoride exposure during critical periods of amelogenesis. DF is characterized by increased porosity (subsurface hypomineralization) with a loss of enamel translucency and increased opacity (Fejerskov *et al*., 1990). It is generally accepted that increasing DF severity correlates with increasing F exposure; however, individual variation in DF severity can exist when F exposure is relatively constant in a community (Mabelya *et al*., 1994; Yoder *et al*., 1998). Ameloblasts in the maturational phase appear to be the cellular target of chronic fluoride exposure (DenBesten and Thariani, 1992), whereas acute fluoride toxicity targets the transitional and early-secretory ameloblasts (Lyaruu *et al*., 2006). The mechanism(s) underlying DF remain obscure, but likely contribute to the observed retention of enamel matrix proteins and may include reduced removal of enamel matrix proteins during enamel maturation, perturbation of extracellular transport, or initiation of the ER stress-response pathway (Matsuo *et al*., 2000; DenBesten *et al*., 2002; Kubota *et al*., 2005; Sharma *et al*., 2008; Bronckers *et al*., 2009; Everett *et al*., 2009). While biological factors likely play critical roles in the pathogenesis of DF, physicochemical affects should also be considered (Robinson *et al*., 2004).

EFFECTS ON HEMATOPOIETIC CELLS AND HEMATOPOIESIS

Fluoride's effects on hematopoietic cells/hematopoiesis have been investigated in *ex vivo* and *in vitro* studies and in different species of mammals. Effects vary depending upon fluoride dose, duration of exposure, and species, and include anemia and leukopenia (Mehdi *et al*., 1978; Eren *et al*., 2005). At modest *in vitro* concentrations (< 500 μM), NaF has been shown to promote the differentiation of human promyelocytic tumor cells (HL-60) to granulocyte-like cells (Kawase *et al*., 1996). Similar exposures of NaF (< 500 μM) can preferentially induce differentiation of primary outbred ddY mouse bone marrow cells along the granulocytic pathway *in vitro* (Oguro *et al*., 2003). These latter studies confirmed differentiation of mouse bone marrow cells along the granulocytic lineage by demonstrating the up-regulation of granulocyte-specific markers (chloroacetate esterase, cell-surface antigens [Mac-1, Gr-1]) with no change in the monocyte-specific markers (non-specific esterase, cell-surface antigens [F4/80, MOMA-2]). The authors hypothesized that any potential shift in differentiation of bone marrow progenitor cells away from the monocytic lineage may affect osteoclast formation.

Evidence that fluoride can affect the bone marrow microenvironment comes from e*x vivo* studies of bone marrow cells collected from several strains of mice treated with NaF in the drinking water. Strain-dependent effects on hematopoietic colony-forming cell unit (CFU) assays were observed (D Yan *et al*., 2007; Chou *et al*., 2009). Bone marrow cells leading to the individual colonies (CFU) may have pluripotent characteristics capable of giving rise to mixed colonies containing multiple hematopoietic lineages (*e.g*., granulocytic, monocytic, and erythroid) or are committed, giving rise to cells from one hematopoietic lineage.

Fluoride treatment of C3H mice shifted hematopoietic differentiation along the monocyte/macrophage lineage, with dose-dependent increases in the frequencies of CFU-GEMM (colony-forming unit granulocyte, erythroid, monocyte/macrophage, and megakaryocyte), CFU-GM (colony-forming unit granulocyte and monocyte/macrophage), and CFU-M (colony-forming unit monocyte/macrophage) colonies. Fluoride's actions on the hematopoietic compartment in the B6 mouse strain differed with a modest increase in the frequency of pluripotential/mixed CFU-GEMM colonies at the highest F dose (100 ppm). The shift in hematopoietic differentiation in the C3H strain correlates with the increase in osteoclast potential observed (D Yan *et al*., 2007). Important precursors for osteoclasts are the CFU-GM and CFU-M.

Mice from the C3H, C57BL/6J (B6), FVB/NJ (FVB), and BALB/cByJ (BALB) strains show strain-specific responses to fluoride in the frequencies CFU-F (colony-forming unit fibroblast) and CFU-OB/Alp+ (colony-forming unit osteoblast/alkaline phosphatase positive) derived from bone marrow cells (Chou *et al*., 2009). The CFU-F are expanded clonal populations that emerge from single bone marrow stromal cells/mesenchymal stem cells (MSC) and possess the potential to differentiate along multiple mesenchymal cell lineages, including osteoblast precursors (Friedenstein *et al*., 1976; Owen and Friedenstein, 1988).

GENETIC STUDIES AND FLUORIDE'S ACTIONS

Until recently, few studies have explored the underlying genetic basis for fluoride resistance or susceptibility. Among the first, high concentrations of fluoride (400 μg/mL) have been used to isolate fluoride-resistant mutants of *Caenorhabditis elegans* (Katsura, 1993). Genetic studies of these mutant nematodes have led to the identification of novel fluoride-resistant (flr) genes, *flr1, flr3*, and *flr4*. The *flr1* gene encodes an ion channel belonging to the degenerin/epithelial sodium channel superfamily, which regulates defecation rhythm (Katsura *et al*., 1994; Take-Uchi *et al*., 1998). The *flr4* gene encodes a predicted

Ser/Thr protein kinase and, like *flr-1,* appears to control rhythmic activities in *Caenorhabditis elegans* (Iwasaki *et al*., 1995; Iwasaki and Thomas, 1997). The *flr-4* gene is closely related to the human *SOK1* gene, a Ste20 protein kinase of the germinal center kinase (GCK) family. The *flr-3* gene remains to be characterized.

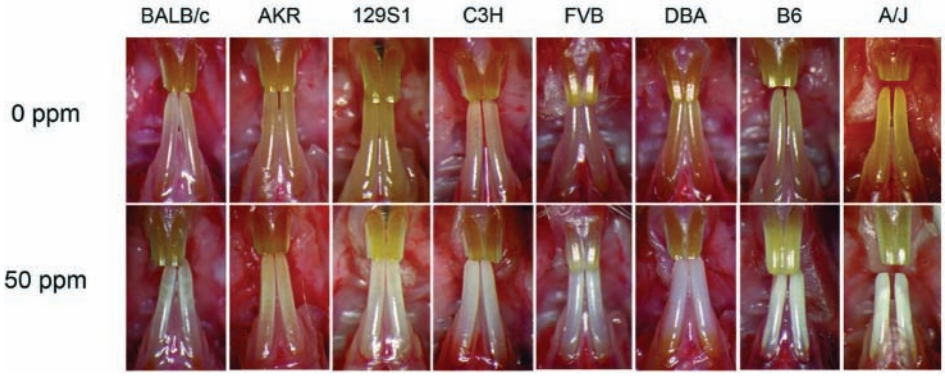

Figure 1. Variation in dental fluorosis severity among inbred strains of mice. Mice at 5 to 6 wks of age were treated with fluoride (0 ppm or 50 ppm [F] ion) in the drinking water for 60 days. All strains developed dental fluorosis. Dental-fluorosis-susceptible strains are on the right, with those more resistant strains on the left side of the panel.

Dental Fluorosis Studies Involving Inbred Strains of Mice

Genetic studies utilizing inbred strains of mice have focused on fluoride's action on tooth enamel development and bone homeostasis (Everett *et al*., 2002, 2009; Vieira *et al*., 2005; Mousny *et al*., 2006, 2008; D Yan *et al*., 2007; Carvalho *et al*., 2009; Chou *et al*., 2009). Inbred mouse strains have been used for genetic studies because of the isogenicity within a strain and the genetic heterogeneity between inbred strains. The genetic diversity existing between inbred strains of mice has yielded phenotypes relevant to human health, such as cancer susceptibility, aging, obesity, susceptibility to infectious diseases, atherosclerosis, blood disorders, and neurosensory disorders (Bogue and Grubb, 2004; Bogue *et al*., 2007; Grubb *et al*., 2009). Humans and mice differ in their dental formulae, and mouse incisors continuously erupt. Despite these differences, mice have been instrumental in our understanding of the important cellular, molecular, and genetic processes controlling odontogenesis. In addition to the genetic diversity between inbred strains of mice, the continuously erupting incisors (active amelogenesis) facilitate the investigation of fluoride's effects on tooth enamel development at any time during the animal's life. Strain-dependent responses to fluoride in the development of dental fluorosis were first demonstrated across 12 inbred strains and the severity of dental fluorosis based upon clinical criteria (tooth enamel appearance) (Everett *et al*., 2002). Genetic diversity and availability were factors in the selection of these 12 strains. From that study, strains clustered into three dental fluorosis groups: resistant strains (129P3/J, FVB/NJ, CBA/J, and DBA/1J); intermediate strains (SWR/J, BALB/cByJ, C57BL/10J, and DBA/2J); and sensitive strains (A/J, SJL/J/ C3H/HeJ, and C57BL/6J). Examples of the variation in dental fluorosis severity are illustrated in Fig. 1. As in humans with DF, clinical criteria can be used to score DF in mice. Since mouse incisors are worn away as they erupt, the DF observed does not reach the deeply pitted and characteristic brown staining seen with severe DF in humans. A modified Thylstrup and Fejerskov (TF) scale can be used to score dental fluorosis in mice (Everett *et al*., 2002, 2009). Alternatively, a modification of quantitative light-induced fluorescence (QLF) (Everett *et al*., 2002; Vieira *et al*., 2005) can be used to provide a more objective means to score dental fluorosis (Fig. 2). Strains (129P3/J, SWR/J, and A/J) representing the three dental fluorosis groups described above were used to show that genetic factors (DF severity) and the environmental factor (fluoride concentration in tooth structure) have similar influence on tooth biomechanical properties, whereas only the environmental factor has an influence on tooth material properties (mineralization) (Vieira *et al*., 2005). Fluoride metabolism also differs between and among mouse strains (Carvalho *et al*., 2009). Whereas the A/J strain consumes more drinking water and required adjustment in [F] to maintain comparable exposure between the two strains, the 129P3/J strain retains more fluoride in the bone and has higher plasma fluoride levels. Despite this important difference, the 129P3/J strain remains resistant to the development of dental fluorosis.

Resistance and susceptibility (risk factors), defined by host and environment interactions, as well as many quantitative phenotypes are considered complex traits. Complex traits (phenotypes) can be assessed quantitatively and are under the control of multiple genes as well as non-genetic (environmental) factors. Multiple genes that contribute to the variation in a phenotypic trait are called quantitative trait loci (QTL). QTLs can be mapped in mice by traditional genetic approaches. Typically, two strains are selected that have widely different traits or responses. The parental mice are then used in a two-generation cross. First F1 hybrid progeny are generated, then used in sister-brother mating to produce F2 mice (Fig. 3). While all F1 mice are genetically identical, each F2 mouse is unique. This is the result of re-arrangement of the parental alleles during gametogenesis (meiotic recombination) in the F1 animals. Mapping of QTLs associated with DF susceptibility was performed with a dental-fluorosis-resistant strain (129P3/J) and the dental-fluorosis-sensitive (A/J) strain in a two-generation cross to create a panel of F2 mice as described above. All F2 mice were treated with fluoride 50 ppm F in the drinking water and, after 60 days, were phenotyped for DF according to the modified TF scale. Treatment of F2 mice with 50 ppm F in the water yields a mean serum [F] of 12.366 ± 1.713 μM. The serum [F] concentrations between F2 mice with different DF severities were not significantly different and were not significantly different from

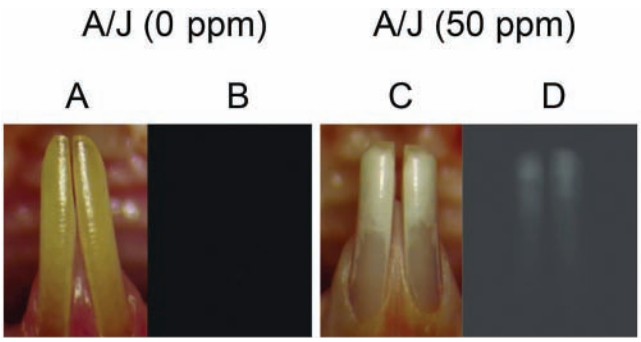

Figure 2. Use of quantitative fluorescence to assess dental fluorosis. Panels **A** and **C** are clinical images of the mandibular incisors from A/J mice treated with control (0 ppm [F]), Panel A; and 50 ppm [F], Panel C) for 60 days. Panels **B** and **D** demonstrate the results of quantitative fluorescence (QF), where a Nikon epifluorescence microscope equipped with a Chroma Gold 11006v2 set cube (Spectra Services Inc., Ontario, NY, USA) (exciter D360/40x, dichroic 400DCLP, and emitter E515LPv2) was used to assess the severity of dental fluorosis. Increased fluorescence is associated with increased dental fluorosis severity.

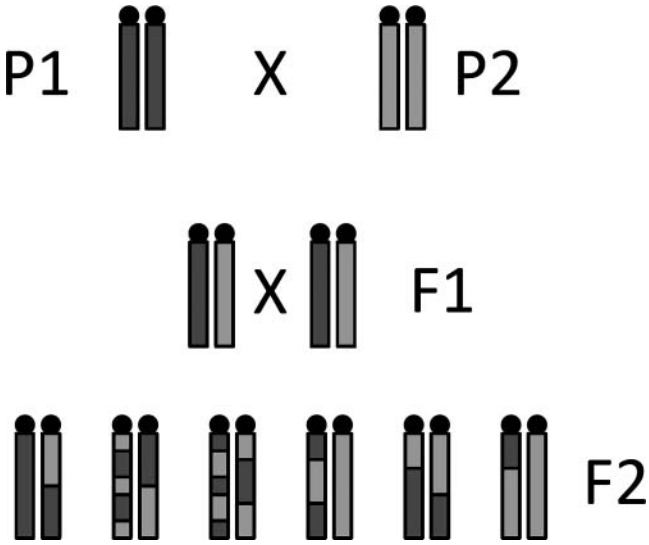

Figure 3. Schematic using inbred progenitor strains in a two-generation cross to produce a panel of F2 progeny. P1 and P2 are progenitor/parental strains that differ in a particular trait of response. The F1 progeny (1st generation) are all genetically identical, having inherited half their genome from P1 and half their genome from P2. The F2 progeny (2nd generation) are composed of genetically unique individuals.

serum [F] concentrations determined in comparably treated parental mice (11.296 ± 3.984 μM). To maximize the power to detect QTLs contributing to the variation in response to dental fluorosis, only the phenotypic extreme F2 animals (those with TF scores of 1 or 4) were genotyped for 354 SNP-based markers distributed throughout the mouse genome. This panel of mice was composed of equal numbers of males and females. Chi-square analysis was performed to compare the genotypic distributions in the two groups of phenotypically extreme F2 mice. Significant evidence of association was observed on chromosomes 2 and 11 for a series of consecutive markers ($p < 0.0001$) (Everett *et al*., 2009). More importantly, there was a lack of significant association on murine chromosomes X, 3, 5, 7, or 9, suggesting little role for amelogenin, ameloblastin, enamelin, amelotin, Klk-4, or Mmp20 in dental fluorosis susceptibility/resistance in this animal model. As illustrated above, the detection and mapping of QTL are straightforward in mice.

Narrowing the QTL intervals to fewer genes and, ultimately, the selection of candidate genes remain the challenging aspects of complex trait dissection. This can be accomplished in mice by increasing marker densities within QTLs and using a complementary approach based upon haplotype mapping. Haplotype Association Mapping (HAM) is a phenotype-driven approach to identify genetic loci in mice. This method is similar to Genome-Wide Association Studies (GWAS) in humans. HAM looks for associations between the phenotype and the haplotypes of mouse inbred strains, treating inbred strains as individuals. Since mice within the strain are isogenic, several individuals can be phenotyped to minimize intra-strain variation. The application of haplotype association mapping in mice was first described in 2001 and has developed into a useful tool for QTL mapping (Grupe *et al*., 2001; Tsaih and Korstanje, 2009). Integrating haplotype-based approaches with traditional mapping tools as described above has great potential for narrowing QTL mapping intervals and prioritizing candidate genes (Pletcher and Wiltshire, 2004; Cervino *et al*., 2005, 2007; Arbilly *et al*., 2006). It is conceivable that interval-specific haplotype analysis based upon an *a priori* knowledge of a QTL interval can reach a resolution of less than 5 Mb (DiPetrillo *et al*., 2005). Recently, haplotype association mapping in mice identified a haplotype block containing the *Cer1* (cerberus 1 homolog) gene that partitions inbred mice strains into high and low bone mineral density groups. The *Cer1* gene is important during embryonic development and appears to play a role in bone development. Based upon the discovery in mice, the human *CER1* gene was investigated, and a non-synonymous SNP (rs3747532) was identified to be associated with increased risk of low bone mineral density in pre-menopausal women (Tang *et al*., 2009).

QTL linkage studies in mice differ from QTL mapping in humans because many human QTL linkage studies are limited in sample size and do not have the family pedigrees that maximize the power to detect linkage (Almasy and Blangero, 2009). High-throughput genotyping and advanced computational analyses have led to the application of genome-wide association studies (GWAS) as a tool for mapping human disease genes (Hindorff *et al*., 2009). As of December 2009, there have been 658 published genome-wide association studies in humans (Hindorff *et al*., 2010).

Bone and Bone Biology Studies Involving Inbred Strains of Mice

Strain-specific responses to fluoride also include effects on bone mineral, bone micro-architecture, and bone cell biology. Bone quality as a function of mechanical properties varies among

three inbred strains (A/J, SWR/J, and 129P3/J) following fluoride exposure (Mousny *et al*., 2006). The A/J strain demonstrated the greatest reduction in bone quality in a fluoride dose-dependent manner. The SWR/J strain demonstrated a modest reduction in bone quality, and the 129P3/J strain was refractory to the effects of fluoride. In an effort to better understand the changes in bone quality observed above, investigators assessed the effects of fluoride on bone formation and mineralization (Mousny *et al*., 2008). All three strains demonstrated an increase in osteoid with the vertebral bodies, which did not translate to increases in bone volume of mineralization.

Mice representing the C3H and B6 inbred strains have been particularly useful in studies of bone biology. In a landmark paper, Beamer and co-workers (1996) investigated peak bone mass in a variety of inbred strains of mice, and with respect to strain differences, the highest value for any given bone parameter was found in the C3H strain, whereas B6 values were absolutely, or statistically, the lowest. These two strains have become the foundation for many investigations contributing to more than 76 scientific articles in the literature, with the majority resulting from studies of bone cells and bone biology. As genetic tools, B6 and C3H mice have contributed to the identification of several quantitative trait loci (QTLs) implicated in determining peak bone mass (Beamer *et al*., 2001; Koller *et al*., 2003; Bouxsein *et al*., 2004). This formed the basis for the selection of C3H and B6 strains for studies involving fluoride. Exposure to fluoride at levels relevant to what might be experienced in humans (Srivastava et al., 1989; Riggs *et al*., 1990) was achieved by treatment of mice with NaF for 3 wks, achieving mean serum [F] of 8 μM (50 ppm F in the water) or 15 μM (100 ppm F in the water).

As discussed above, fluoride can have effects on the bone marrow microenvironment. The bone marrow provides a source for osteoprogenitors as well as osteoclast precursors. In addition to the increased osteoclast potential in C3H mice following fluoride treatment, there are concurrent changes in markers for markers of osteoclast activity *in vivo* (D Yan *et al*., 2007). Fluoride induces intact parathyroid hormone (iPTH), soluble receptor activator for nuclear factor kappa B ligand (RANKL), and osteoclast-specific tartrate-resistant acid phosphatase (Trap5b) in the serum of C3H mice. PTH conveys effects on osteoblast cells, which in turn produce factors like RANKL. PTH is an important early step in osteoclastogenesis. It is also worth noting that there have been repeated associations between increased fluoride intake and increases in circulating PTH and hyperparathyroidism (Sivakumar and Krishnamachari, 1976; Ream and Principato, 1981; Gupta *et al*., 2001; Chadha and Kumar, 2004). Stronger evidence of enhanced osteoclastogenesis is the increase in the number of osteoclasts along the bone surfaces in C3H mice. B6 mice respond quite oppositely (D Yan *et al*., 2007). In this strain, the anabolic actions of fluoride are favored. There is an increase in total alkaline phosphatase (ALP) in the serum, with a concomitant decrease in the pro-osteoclastogenesis markers iPTH and RANKL. Finally, there is a modest increase in trabecular bone BMD and reduction in bone quality in B6 mice. A summary of the differential actions of fluoride on B6 and C3H mice is shown in Fig. 4.

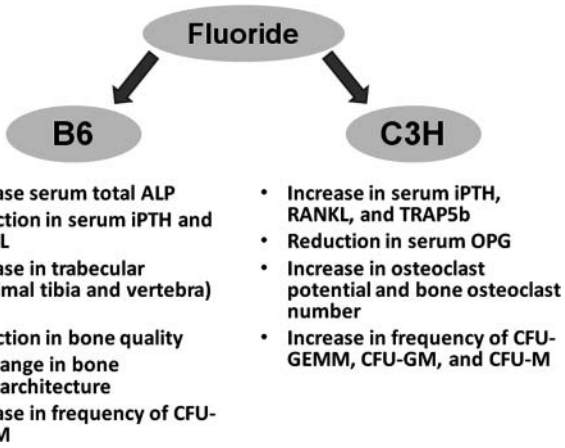

Figure 4. Dichotomy of fluoride responses between the high-bone-mass C3H/HeJ and low-bone-mass C57BL/6J inbred strains. The C57BL/6J strain favors the anabolic actions of fluoride, whereas the C3H/HeJ strain demonstrates enhanced osteoclastogenesis.

As mentioned earlier, C3H and B6 mice differ in their peak bone mass. Many other notable differences in bone and bone biology exist between these two strains. In particular, bone adaptation responses in B6 mice are greater than those in C3H (Akhter et al., 1998, 2002; Amblard *et al*., 2003), and bone loss due to disuse is also greater in B6 mice (Judex *et al*., 2004). C3H mice operate at a high-bone-mass production mode, where perhaps the anabolic actions of fluoride are not favored as in B6 mice. Mapping studies similar to that undertaken to identify DF-associated QTLs can be undertaken with B6 and C3H mice as progenitor strains to identify QTLs associated with fluoride's actions on osteoclastogenesis.

SUMMARY

Fluoride in various chemical forms, doses, and exposures has physicochemical and biologic effects on cells and tissues. A narrow therapeutic/toxicity window and biphasic actions further complicate our understanding of fluoride's effects. Fluorides mediate their actions through MAPK signaling pathways, leading to changes in gene expression, cell stress, and even cell death. Fluorides can lead to a diverse collection of responses affecting biomineralization. The role that an individual's genetic background plays in modulating fluoride's actions is becoming more evident and will allow for the investigation of gene-gene and gene-environment interactions capable of modifying the function(s) of fluoride-responsive genetic variants in an animal model. This in turn will provide a better understanding of the effects of fluoride on human bone, bone cells, and tooth enamel development. Future studies will likely focus on identifying and characterizing fluoride-responsive genetic variations (*e.g*., polymorphisms), and on identifying those at-risk human populations who are susceptible to the unwanted or potentially adverse effects of fluoride action, and, finally, on elucidation of the fundamental mechanisms by which fluoride affects biomineralization.

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