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A MODEL FOR THE PHARMACOLOGICAL TREATMENT OF CROUZON SYNDROME

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Abstract

OBJECTIVE—Crouzon syndrome is caused by mutations in fibroblast growth factor receptor 2 (*FGFR2*) leading to constitutive activation of receptors in the absence of ligand binding. The syndrome is characterized by premature fusion of the cranial sutures that leads to abnormal cranium shape, restricted brain growth, and increased intracranial pressure. Surgical remodeling of the cranial vault is currently used to treat affected infants. The purpose of this study was to develop a pharmacological strategy using tyrosine kinase inhibition as a novel treatment for craniosynostotic syndromes caused by constitutive FGFR activation.

METHODS—Characterization of cranial suture fusion in *Fgfr2*^{C342Y/+} mutant mice, which carry the most common Crouzon mutation, was performed using microcomputed tomographic analysis from embryogenesis through maturation. Whole calvarial cultures from wild-type and *Fgfr2*^{C342Y/+} mice were established and cultured for 2 weeks in the presence of dimethyl sulfoxide control or PD173074, an FGFR tyrosine kinase inhibitor. Paraffin sections were prepared to show suture morphology and calcium deposition.

RESULTS—In untreated *Fgfr2*^{C342Y/+} cultures, the coronal suture fused bilaterally with loss of overlap between the frontal bone and parietal bone. Calvaria treated with PD173074 (2 μmol/L) showed patency of the coronal suture and were without evidence of any synostosis.

CONCLUSION—We report the successful use of PD173074 to prevent in vitro suture fusion in a model for Crouzon syndrome. Further studies are underway to develop an in vivo treatment protocol as a novel therapeutic modality for *FGFR* associated craniosynostotic syndromes.

Keywords

Craniosynostosis; Crouzon syndrome; Fibroblast growth factor receptor 2; PD173074; Tyrosine kinase inhibitor

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Understanding the normal and abnormal molecular mechanisms of development and disease has become the defining theme of medicine in the 21st century. In the field of craniofacial surgery, genetic and developmental studies have elucidated the etiology of many common anomalies. One such set of conditions is faciocraniosynostoses, a group of closely related craniosynostotic syndromes caused by mutations in the genes encoding the fibroblast growth factor receptors (FGFRs). Crouzon syndrome was first described nearly 100 years ago when the triad of calvarial deformities, facial anomalies, and exophthalmos was noted in a mother and her son (10). The syndrome is autosomal dominant with an incidence of approximately 1:25,000 to 1:60,000 births (5,7). Premature fusion of the cranial sutures is the hallmark of the condition, with craniosynostosis most commonly occurring at the coronal sutures bilaterally, resulting in a turribrachycephalic deformity. Fusion of the sagittal and lambdoid sutures is also noted frequently (11).

The molecular basis of Crouzon syndrome was identified in 1994 (22,29). Reardon et al. (29) found single-stranded conformational polymorphism variations in exon 9 of *FGFR2* in nine individuals with Crouzon syndrome. In five of the nine patients, a replacement of a cysteine in an immunoglobulin-like domain was seen. To date, more than 30 mutations have been found to cause Crouzon syndrome alone, most of which are localized to the immunoglobulin III domain of *FGFR2* (6). Interestingly, identical mutations in the *FGFR2* gene cause both Crouzon and Pfeiffer syndrome phenotypes (31). Pfeiffer syndrome also presents with craniosynostosis but can be differentiated from Crouzon syndrome clinically by broad, medially deviated great toes and thumbs with or without syndactyly (28).

FGFR2 is one of four signaling FGFRs belonging to the receptor tyrosine kinase family (15). Mutations in *FGFRs* lead to abnormal cell signaling through one of three mechanisms: 1) increased receptor signaling caused by increased ligand affinity (1,2,36); 2) activation by removal of inhibition by an activating loop in the kinase domain (33); or 3) receptor dimerization by unpaired cysteines, leading to constitutive receptor activation in the absence of ligand binding (9,25,35). Trans-membrane hydrogen bonding also has been reported (35). In Crouzon and, in many cases, Pfeiffer syndromes, the mechanism of increased receptor activation is via receptor dimerization by unpaired cysteines (21).

To understand the biological effects of FGFR2 hyperactivation, Iseki et al. (16) placed fibroblast growth factor 2-soaked beads over the developing coronal suture in mice using an ex utero technique. This demonstrated that excessive fibroblast growth factor-FGFR interaction leads to down-regulation of *Fgfr2* and up-regulation of osteopontin, a marker for differentiation (16,17). To further explore the effects of constitutive activation of FGFR2 on craniofacial growth, Eswarakumar et al. (13) created a Crouzon/Pfeiffer syndrome mouse. The model was made using site-directed mutagenesis in which the Cys342 “TGC” was changed to “TAC” on a *Fgfr2* genomic fragment containing exon 9. The mutation represents the most commonly encountered mutation in individuals with Crouzon syndrome. *Fgfr2*^{C342Y/+} mice are born viable, and as growth progresses, they develop a rounded calvaria, proptotic eyes, and shortened midface (13,26). Fusion of the coronal sutures occurs bilaterally, with partial to complete fusion of the sagittal and lambdoid sutures also occurring. Low levels of FGFR2 have been confirmed in fused cranial sutures from human patients with Crouzon syndrome (3). In the developing suture, down-regulation of *Fgfr2* after stimulation in FGF2 has been correlated with a decrease in cell proliferation and, interestingly, an up-regulation of *Fgfr1*, leading to enhanced differentiation of osteoprogenitor cells (13,17). This drive toward osteogenic differentiation in the uncommitted cells of the suture has been implicated as the mechanism behind the abnormal suture fusion that defines the craniosynostotic conditions (24,35).

Current management of infants born with syndromic craniosynostosis is based on surgical remodeling of the fronto-orbital region to prevent functional disturbances and enable normal development and shape of the cranium (30). In addition, midface advancement, whether by conventional techniques or distraction osteogenesis, may be required to treat the hypoplastic midface. The development of the *Fgfr2*^{C342Y/+} mouse, however, has led to our ability to investigate nonsurgical interventions that may prevent suture fusion and normalize craniofacial growth in Crouzon/Pfeiffer syndrome. PD173074, a pyrido-[2,3-d]pyrimidine, is a selective FGFR tyrosine kinase inhibitor (23). On the basis of our understanding of altered suture biology, we hypothesized that the use of PD173074 could mitigate the biochemical effects of the Crouzon/Pfeiffer mutation by attenuating the drive toward premature differentiation seen in the osteoprogenitor cell population of affected cranial sutures. The purpose of this study was to develop a pharmacological strategy using tyrosine kinase inhibition as a novel treatment for craniosynostotic syndromes caused by constitutive FGFR activation.

METHODS

Animals

Fgfr2^{C342Y/+} mutant mice were a gift of the late Professor Peter Lonai (Weizmann Institute, Israel) and were constructed as described by Eswarakumar et al. (13). All procedures were carried out in accordance with the guidelines of the Animal Studies Committee of the Washington University School of Medicine. Male mice that were heterozygous for the *Fgfr2*^{C342Y} mutation were bred with CD1 wild-type (WT) females. Timing of the embryos was by the vaginal plug method, with 12:00 noon on the day on which the plug was observed referred to as 0.5 days past coitum. The WT versus mutant genotype was determined with polymerase chain reaction amplification of the WT and mutant allele.

Micro-computed Tomography

For three-dimensional computed tomographic (CT) scanning, 5 WT and 5 *Fgfr2*^{C342Y/+} specimens were obtained at the following stages of development: embryonic Day (E) 17.5, postnatal day (P)1, P14, P28, and P42. They were then sealed in conical tubes and shipped to the MicroCT imaging facility at the University of Utah. The use of MicroCT for analysis of mutant mouse models is established (14). We also have validated data from MicroCT scans of *Fgfr2*^{C342Y/+} specimens by comparison with skeletal preparations (26). Images were obtained at 32 μ m resolution using a General Electric Medical Systems EVS-RS9 MicroCT scanner (Chalfont St. Giles, England). Image data then were sent to the Craniofacial Imaging Laboratory (27) at the St. Louis Children's Hospital and to the 3D Imaging Laboratory at the University of Copenhagen School of Dentistry and processed using contemporary medical imaging software for analyses of the craniofacial skeleton.

Calvarial Culture System

Pregnant females at E 18.0 were sacrificed by CO₂ asphyxiation, and embryos were dissected from the uterus under sterile conditions. With the aid of the dissecting microscope, the skin was removed from the overlying cranium, and a circumferential incision was made to carefully remove the calvarium. The brain was then removed, leaving the dura adherent to the calvarium. Each calvarium was placed into a well of a culture plate over a 400 μ l mound of Matrigel (BD Biosciences, Bedford, MA) placed at the bottom of each well. This allowed the calvarium to maintain its shape and remain above the plate bottom. Calvaria were cultured for 2 weeks in 1 ml of Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 μ g/ml ascorbic acid, 1% penicillin/streptomycin/ amphotericin, and 2 mmol/L L-glutamine, in an incubator at 37°C in a 95% air/5% CO₂ environment. The medium was changed every 48 hours. After it was established that the timing and pattern of suture fusion in the mice calvaria cultures was consistent with the in vivo patterns of suture

fusion seen by MicroCT, the efficacy of PD173074 (courtesy of Pfizer, Morris Plains, NJ) to prevent suture fusion was tested. The experiment was repeated in triplicate with three specimens per group. Group 1 consisted of WT specimens treated with 0.3% dimethyl sulfoxide (DMSO) carrier. Group 2 contained WT specimens treated with 2 μ mol/L PD173074 dissolved in DMSO. Group 3 contained *Fgfr2*^{C342Y/+} specimens treated with 0.3% DMSO. Group 4 contained *Fgfr2*^{C342Y/+} specimens treated with 2 μ mol/L PD173074 in DMSO. After 14 days in culture, calvaria were fixed in 10% buffered formalin, and paraffin sections were prepared and stained with Mallory's trichrome and von Kossa stain to show suture morphology and calcium deposition.

RESULTS

Suture Morphology

Patterns of craniofacial dysmorphology were examined from an embryonic stage (E 17.5) through maturation (6 wk) using two- and three-dimensional (3-D) MicroCT. *Fgfr2*^{C342Y/+} mice showed a craniofacial phenotype very similar to that of affected humans, with complete fusion of the coronal sutures and varying degrees of lambdoid and sagittal suture fusion (Fig. 1). Midface hypoplasia and a Class III malocclusion also was present. As shown in Figure 2, the brachycephaly associated with the Crouzon phenotype was not readily apparent until 2 weeks of age. This change in head shape was found to correlate with fusion of the calvarial sutures, particularly the coronal sutures. Cranial sutures were present just before birth and patent in all specimens. There was no evidence of agenesis of any suture (Fig. 3). No complete suture fusion was found in any specimen before the 2-week-old stage. However, at the 2-week-old stage, coronal suture fusion was noted in all specimens. This indicates that in *Fgfr2*^{C342Y/+} mice, a postnatal window of opportunity exists in which the sutures remain patent, and pharmacological therapy could have a preventative effect.

Pharmacological Inhibition of FGFR In Vitro

To assess the effects of FGFR tyrosine kinase inhibition on postnatal suture fusion in *Fgfr2*^{C342Y/+} mice, the kinase inhibitor PD173074 was used to treat cultured calvarial complexes (osseous calvarium plus dura). Changes in suture morphology and patency was compared in untreated calvaria and calvaria treated with the inhibitor to identify a therapeutic response. In WT cultures, a normal overlap between the frontal bone and the parietal bone was seen at the site of the coronal suture, with no evidence of suture fusion (Fig. 4). In untreated *Fgfr2*^{C342Y/+} specimens, the coronal suture could not be visualized because synostosis of the frontal and parietal bone resulted in the loss of any discernable suture. However, calvaria treated with PD173074 (2 μ mol/L) showed patency of the coronal suture and was without evidence of synostosis (Fig. 4). The normal overlap between the frontal bone and the parietal bone remained intact, although there appeared to be some blunting of the bones at their overlapping edges. The overall size and shape of the calvarium was unchanged from WT controls. There was no effect of the drug or DMSO carrier on the sutures of WT specimens. (Fig. 5)

DISCUSSION

We report the successful use of PD173074 to prevent suture fusion in a model for Crouzon syndrome. PD173074 exhibits a high degree of complementarity with the tyrosine kinase domain of the FGFR tyrosine kinase domain, particularly FGFR1 (23). PD173074 has been used successfully to inhibit ectopic or excessive FGFR signaling in other human diseases, including thyroid carcinoma, myeloma, breast cancer, and as an antiangiogenic agent for tumor chemotherapy (12,18,32,34). At first glance, use of an FGFR inhibitor appears paradoxical, given the down-regulation of *FGFR2* demonstrated in human and murine experiments (3,13,16). However, our intention in developing this therapy was to attenuate the premature

differentiation of osteoprogenitor cells at the leading bone edges (i.e., the cranial sutures), which has been correlated with concurrent up-regulation of *Fgfr1* (19). Further investigation into the specific mechanism of PD173074 on suture patency currently is underway in our laboratory.

Given the results of these calvarial culture studies, we know that pharmacological inhibition of suture fusion is possible in a murine model for Crouzon and Pfeiffer syndromes. This poses several questions that must be addressed. Will this compound be effective in an in vivo application when given to live mice with the Crouzon mutation? Can it be given prenatally? What will be the best mechanism of drug delivery to maximize therapeutic effectiveness and limit potential side effects? Is there a role for limited usage, such as directly applying the compound to the sutures via a controlled release system? Last, and perhaps most critical, is this a realistic therapy for human infants affected with *FGFR*-associated syndromic craniosynostosis? The basis of this question lies in the timing of premature suture fusion in humans, which traditionally has been considered a prenatal event because infants are diagnosed during early infancy. However, multiple reports exist describing patients in which the Crouzon syndrome calvarial phenotype developed during early infancy (4,8,11). In these cases of progressive postnatal craniosynostosis, infants develop the midface characteristics of Crouzon syndrome soon after birth but show delayed, progressive craniosynostosis and increased intracranial pressure. In this subtype of patients, pharmacological intervention certainly could have a beneficial outcome. As for those patients diagnosed with the condition at or shortly after birth, perhaps a drug such as PD173074 could be used as an adjuvant to surgical therapy by preventing further suture fusion of the vault, endocranial base, and midface. This may reduce the growth disturbances seen with the syndrome and limit the frequency or invasiveness of surgical interventions. In addition, sonographic features of Crouzon syndrome are readily apparent during prenatal ultrasound (20) and perhaps initiation of *FGFR* tyrosine kinase inhibition at this early stage could further improve outcomes. These will be difficult questions to answer but well worthwhile, given the limited alternatives for treatment currently in practice.

In conclusion, we report the successful use of an *FGFR* tyrosine kinase inhibitor to prevent suture fusion in a mouse model for Crouzon and Pfeiffer syndromes. Further work will elucidate the molecular effects of this compound on suture behavior and move toward the development of a possible therapeutic intervention for humans affected with these conditions.

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Figure 1.

A, 3-D CT scan of a normal infant showing unfused coronal suture (C). B, 3-D CT scan of an infant with Crouzon syndrome showing fused coronal suture with visible ridging (arrow), abnormal head shape (brachycephaly), and midface hypoplasia. C, 3-D CT scan of a 6-week-old wild-type (WT) mouse showing unfused coronal (C), sagittal (S), and lambdoid (L) sutures. D, 3-D CT scan of a 6-week-old *Fgfr2*^{C342Y/+} mouse showing craniosynostosis of coronal and sagittal sutures. The lambdoid sutures remain open. E, sagittal section of two-dimensional CT showing patency of coronal suture in WT mice (inset). F, similar view of *Fgfr2*^{C342Y/+} mouse illustrating the fusion of the coronal suture (inset).

Figure 2. Timeline of craniofacial dysmorphology of *Fgfr2^{C342Y/+}* mice. Brachycephalic appearance does not appear until 2 weeks of age, which correlates with the fusion of the coronal sutures in *Fgfr2^{C342Y/+}* mice.

Figure 3. Patency of the coronal sutures at E18.5, just before birth. A, wild-type specimen. B, *Fgfr2*^{C342Y/+} specimen. The image is inverted surface reconstruction of 3-D CT data. The coronal suture is indicated by an asterisk between the frontal bone (F) and parietal bone (P).

Figure 4. von Kossa staining showing calcium deposition in sagittal sections through calvarial cultures. A, WT mouse calvarium after 14 days in culture. The line indicates the plane of section for B to D (scale bar = 2.0 mm). B, WT coronal suture after 14 days in culture. The normal overlap of frontal and parietal bones is seen with patency of the suture. C, fused coronal suture in *Fgfr2*^{C342Y/+} specimen treated with DMSO control. Note the ridging of the bone, which is similar to that shown in Figure 1B (black arrow). D, treatment of *Fgfr2*^{C342Y/+} calvarium with PD173074 prevents suture fusion (gray arrow) (scale bar = 300 μ m).

Figure 5.

Normal suture development is regulated in part by FGFRs that control proliferation-differentiation rate of osteogenic stem cells (A). In Crouzon syndrome, activation of FGFR2 leads to acceleration of differentiation process with loss of proliferating stem cells at the suture front and subsequent suture fusion (B). Treatment with PD173074 prevents abnormal suture obliteration.