Increased Calvaria Cell Differentiation and Bone Matrix Formation Induced by Fibroblast Growth Factor Receptor 2 Mutations in Apert Syndrome

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Abstract

Apert syndrome, associated with fibroblast growth factor receptor (FGFR) 2 mutations, is characterized by premature fusion of cranial sutures. We analyzed proliferation and differentiation of calvaria cells derived from Apert infants and fetuses with FGFR-2 mutations. Histological analysis revealed premature ossification, increased extent of subperiosteal bone formation, and alkaline phosphatase–positive preosteoblastic cells in Apert fetal calvaria compared with age-matched controls. Preosteoblastic calvaria cells isolated from Apert infants and fetuses showed normal cell growth in basal conditions or in response to exogenous FGF-2. In contrast, the number of alkaline phosphatase–positive calvaria cells was fourfold higher than normal in mutant fetal calvaria cells with the most frequent Apert FGFR-2 mutation (Ser252Trp), suggesting increased maturation rate of cells in the osteoblastic lineage. Biochemical and Northern blot analyses also showed that the expression of alkaline phosphatase and type 1 collagen were 2–10-fold greater than normal in mutant fetal calvaria cells. The in vitro production of mineralized matrix formed by immortalized mutant fetal calvaria cells cultured in aggregates was also increased markedly compared with control immortalized fetal calvaria cells. The results show that Apert FGFR-2 mutations lead to an increase in the number of precursor cells that enter the osteogenic pathway, leading ultimately to increased subperiosteal bone matrix formation and premature calvaria ossification during fetal development, which establishes a connection between the altered genotype and cellular phenotype in Apert syndromic craniosynostosis. (*J. Clin. Invest.* 1998. 101:1310–1317.) Key words: calvaria • Apert syndrome • craniosynostosis • osteogenesis • fibroblast growth factor receptor 2 mutations

Introduction

Apert syndrome is an autosomal dominant syndrome characterized by severe bicoronal craniosynostosis and other abnormalities occurring at lower frequency (1). More than 98% of hitherto reported cases were ascribed to recurrent mutations in the fibroblast growth factor receptor (FGFR)[1] 2 gene (2). Mutations in FGFR-2 also cause Pfeiffer, Jackson-Weiss, and Crouzon syndromes, and mutations in FGFR-1 were found in Pfeiffer syndrome (3–7). FGFRs are composed of a unique transmembrane domain, an extracellular domain composed of three Ig-like domains, and an intracellular domain containing two tyrosine kinase subdomains (8, 9). In patients with Apert syndrome, mutations in exon IIIa of FGFR-2 produce missense substitutions involving adjacent amino acids in the linker region between the second and third extracellular Ig domains (2). Similar mutations were shown to cause ligand-independent activation of the receptor in FGFR-3 (10). Constitutive receptor activation of FGFR-2 was also demonstrated in Crouzon syndrome (11, 12). Although FGFR mutations may induce abnormalities in the proliferation or differentiation of cells involved in suture closure and bone formation (9, 13), the cellular abnormalities brought about by Apert FGFR-2 mutations in human calvaria osteogenic cells are not known.

Several observations indicate that FGFs control bone cell function during early bone formation. FGF-2 is a potent mesodermal inducer during skeletal development (14). In addition, FGF-2 stimulates osteoblastic cell proliferation (15–17) and inhibits alkaline phosphatase (ALP) activity, and collagen type 1 (Col 1) and osteocalcin (OC) expression (17–19). In contrast, FGF-2 increases the number and differentiation of osteogenic precursor cells in the bone marrow stroma (20–23), and increases endosteal bone formation in vivo (24, 25). Therefore, identification of the phenotypic consequences of FGFR-2 mutations in bone-forming cells would be an important step in understanding the role of FGF-2 and FGFR-2 during membranous ossification in humans.

Using a new model of human neonatal calvaria cell culture (26), we showed previously that the premature calvarial ossification in children with nonsyndromic, isolated primary stenosis is associated with increased calvaria cell differentiation (27). In this study, we determined the phenotypic consequences of Apert FGFR-2 mutations on human calvaria cell proliferation and differentiation.

Methods

Subjects and bone samples. Calvaria bone samples were obtained from two infants, aged 5 and 5.5 mo, and four fetuses, aged 19–28 wk,

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1. *Abbreviations used in this paper:* ALP, alkaline phosphatase; Ap, Apert; Co, control; Col 1, collagen type 1; FGF, fibroblast growth factor; FGFR, FGF receptor; im, immortalized; OC, osteocalcin; P1CP, carboxy-terminal propeptide; rh, recombinant human.

with clinical evidence of Apert syndrome (see Table I). Normal calvaria bone samples were obtained from 10 infants, aged 5–6 mo, who underwent a local reconstruction of the skull for nonsyndromic, isolated primary stenosis, or for tumor resection, according to the French Ethical Committee recommendations (27). In these control subjects, samples were obtained from normal, nonstenosed bone at equivalent areas. Normal fetal calvaria bone samples were obtained from six aborted fetuses, aged 19–26 wk, with no evidence of bone disease. The samples consisted mostly of trabecular bone, with more trabecular bone in Apert subjects than in controls, because of the premature calvaria ossification. The sutures (saggital, metopic, or coronal) were tiny in controls, or fused in Apert subjects. The bone samples were sectioned into two parts and used for histological and cell culture studies (see Table I).

Mutation analysis. Blood or tissue samples were collected from all subjects with Apert syndrome. Genomic DNA was extracted from lymphocytes by SDS lysis, proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation before resuspension in Tris-EDTA, according to standard procedures (6). FGFR-2 was screened for mutations in exon IIIa by fluorometric sequencing or restriction digestion with MboI and BglI (6).

Histological analysis. For histology, the calvaria sample in the four fetuses with Apert syndrome (see Table I) and in three age-matched control fetuses was fixed in 70% ethanol and embedded undecalcified in methyl or glycol methacrylate (28). Longitudinal sections (5 μm thick) of plastic-embedded bone samples were stained with Goldner trichrome or von Kossa staining to identify the mineralized matrix, or with ALP staining (28) to stain osteoprogenitors, preosteoblasts, and osteoblastic cells. The total amount of calcified bone matrix (fraction of the total bone area) and the fraction of subperiosteal bone surface with bone matrix formation, osteoid and osteoblasts (expressed as percentage of the total bone surface) were measured on Goldner-stained sections by conventional histomorphometric methods (29).

Human calvaria cell cultures. Calvaria cells were obtained from bone samples from two Apert infants and two Apert fetuses (see Table I), and from age-matched control infants ($n = 10$) and fetuses ($n = 6$). In each sample, calvaria cells were obtained by collagenase digestion as described previously (26, 27). Briefly, the samples were dissected into small fragments, washed extensively with PBS to remove marrow cells, treated with 0.25% collagenase (Type I; Sigma Chemical Co., St. Louis, MO) for 2 h at 37°C, washed in DME supplemented with glutamine (292 mg/liter), 10% heat-inactivated FCS, and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), collected by centrifugation, and cultured in DME with 10% FCS until confluence. This method yields a cell population with characteristics of preosteoblastic cells (26). No hematopoietic or adipocytic cell was present in the cultures. Calvaria cells obtained from one 25-wk-old Apert fetus (see Table I), called Apert (Ap) fetal cells, and control (Co) fetal cells from one age-matched control fetus were expanded to study the cellular phenotype in greater detail. Ap cells expressed the Ser252Trp mutation, the most frequent mutation in Apert syndrome (2), as found by PCR and restriction enzyme analyses (data not shown).

Analysis of calvaria cell growth. Cell growth under basal conditions was evaluated as described (30) in calvaria cells obtained from one to four samples per individual in two infants and two fetuses with Apert syndrome (see Table I), and from ten control infants and six control fetuses of the same age. Cells plated at 10,000 cells/cm^2 were labeled with 2 μCi/well of 6-[^{3}H]thymidine on days 0, 2, 6, and 13, and [^{3}H]thymidine incorporation into DNA was determined 24 h later in three aliquots by liquid scintillation counting. The growth curve was derived from the growth profile, and the maximal DNA synthesis (higher value for [^{3}H]thymidine incorporation) and total DNA synthesis activity (cumulative peak of [^{3}H]thymidine incorporation into DNA at 14 d of culture) were determined (30).

Analysis of calvaria cell differentiation. ALP activity and osteocalcin (OC) production, two markers of cells in the osteoblastic lineage, were determined under basal conditions in calvaria cells obtained separately from one to six samples per individual in two infants and one fetus with Apert syndrome (see Table I), and in age-matched control infants ($n = 8$) and fetuses ($n = 5$). Cells plated at 10,000 cells/cm^2 were cultured in DME with 10% FCS until confluence, washed, then cultured in serum-free medium for 48 h in the presence of vitamin K_1 and 50 μg/ml ascorbic acid. OC levels in the medium were measured by RIA (Cis bio, Gif sur Yvette, France) (26, 27). ALP determination in cultured cells was performed as described (26, 27).

To evaluate the fraction of calvaria cells committed to the osteogenic phenotype, a histochemical staining of ALP was performed in Ap and Co fetal cells. Cells cultured at subconfluence were rinsed with cold PBS, fixed in 70% ethanol at 4°C, and incubated for 1 h at 37°C with naphthol AS-BI phosphate in Tris buffer (pH 8.5) in the presence of Fast Red Violet LB salt. The number of ALP-positive Ap and Co cells (percentage of total number of cells) was quantified at a magnification of 125 on 20 fields (> 4,000 cells).

Response of mutant calvaria cells to FGF-2. The effects of exogenous FGF-2 on cell growth and on markers of differentiation were examined in Ap fetal cells with the Ser252Trp mutation and in Co fetal calvaria cells. Ap and Co cells plated at 10,000 cells/cm^2 and cultured until preconfluence for cell proliferation, or to confluence for cell differentiation, were depleted of serum for 48 h and treated with recombinant human (rh)FGF-2 (PeproTech, Inc., Rocky Hill, NJ) at different concentrations (0.1–10 ng/ml) for 48 h. Cell growth was determined by DNA synthesis as described above, and by counting actual cell number after cell detachment with trypsin. ALP activity and OC released into the medium were determined as described (26, 27). The production of Col 1 was evaluated by determining the levels of carboxy-terminal propeptide (P1CP) released into the medium, which reflect Col 1 synthesis by human osteoblastic cells. P1CP levels were measured with a procollagen ^{125}I-RIA kit (Orion Diagnostica, Espoo, Finland) with intra- and interassay variability of 3.2 and 4.0%, respectively.

Analysis of gene expression in mutant calvaria cells. Since a large amount of cells is required for evaluation of gene expression, we immortalized Ap and Co cells using a plasmid containing the SV-40 large T antigen (31). The nonimmortalized cells were eliminated, only immortalized cells were selected according to the rate of cell growth, and the entire population of immortalized cells was used for further studies. Immunocytochemistry and reverse transcription PCR showed genomic functional insertion of large T antigen in immortalized cells (data not shown). The expression of ALP and type I collagen mRNA was determined by Northern blot analysis in immortalized (im) Ap and imCo fetal cells in basal culture condition. Total cellular RNA was extracted from confluent imAp and imCo cells using Extract-All solution according to the manufacturer's instructions (EuroBio S.A., Les Ulis, France). 20 μg of RNA per lane was separated on 1.2% formaldehyde/agarose gel and transferred to nylon filter in 0.05 M NaOH for 3 h. The filter was hybridized with cDNA probes encoding ALP and Col 1 genes, stripped, and rehybridized with 18S probe. Inserts were labeled with [α-^{32}P]dCTP using a nick translation kit (Promega, Charbonnières les Bains, France). Hybridization was carried out overnight at 42°C in the same buffer with the addition of 10^6 cpm of radiolabeled probe per milliliter. The filter was washed twice at room temperature in 2× SSC/0.1% SDS for 15 min, then once at 65°C in 0.1× SSC/0.1% SDS (high stringency conditions) for 30 min.

Osteogenic capacity of mutant calvaria cells. The osteogenic capacity of imAp and imCo cells was determined by plating the cells at high density (2×10^5–1×10^6 cells) on bacteriological grade dishes that induce the formation of aggregates and osteogenesis in immortalized osteogenic cells (32). Only small aggregates were formed in order to avoid the problem of lack of nutrients and possible occurrence of dystrophic calcification in the center of the aggregates. After 2 wk of culture in the presence of 50 μg/ml ascorbic acid and 3 mM phosphate (32), the aggregates were harvested, fixed in 70% ethanol,

Table I. FGFR-2 Mutations in Apert Subjects and Experiments Performed on Calvaria Samples

Patient age*	Mutation	Tissue studies	Cell culture studies
Infants			
5.0 M	Pro253Arg	—	Growth, differentiation markers
5.5 M	Ser252Trp	—	Growth, differentiation markers
Fetuses			
19 W	Ser252Trp	Histology	—
25 W	Ser252Trp	Histology, histochemistry	Growth, differentiation markers, mRNA analysis, in vitro osteogenesis
27.5 W	Ser252Trp	Histology	—
28 W	Ser252Phe	Histology, histochemistry	Growth

*All patients had a clinical phenotype of Apert syndromic craniosynostosis. *M*, Months. *W*, Weeks.

and embedded undecalcified in glycol methacrylate (33). Thin (5 μm) histologic sections were stained with Goldner trichrome to visualize matrix condensation and osteogenesis, or with von Kossa stain for mineral detection (28, 33).

Statistics. The data were expressed as the mean±SEM. Differences between the mean values were analyzed using the statistical package super-ANOVA (for Macintosh; Abacus Concepts, Inc., Berkeley, CA) with a minimal significance of $P < 0.05$.

Results

Mutation analysis. FGFR-2 mutations were determined in the whole series of Apert patients. Sequencing of genomic DNA revealed a Pro253Arg mutation in one infant and a Ser252Phe in one fetus. Others had the Ser252Trp mutation (Table I).

Calvaria of Apert fetuses show increased subperiosteal pre-

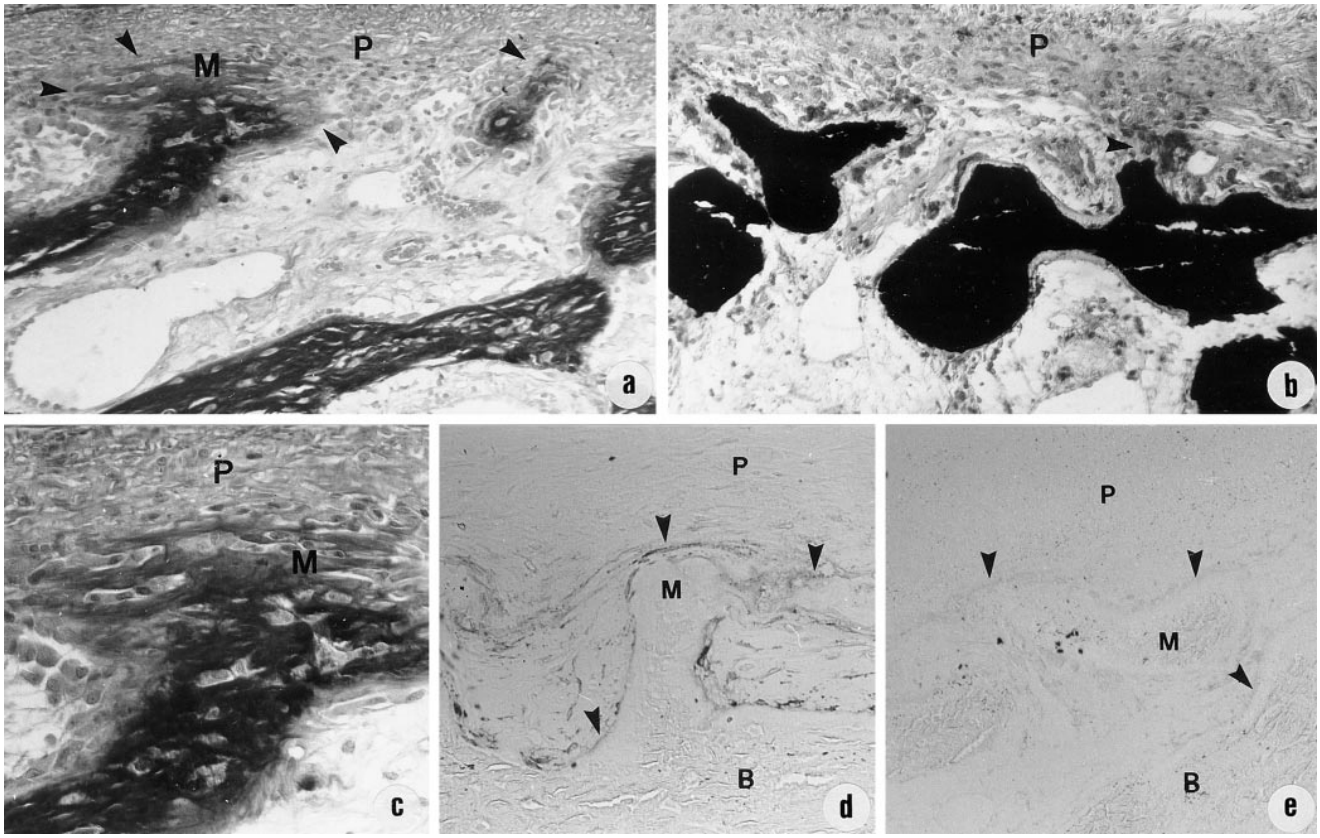

Figure 1. Histological aspect of the subperiosteal area in calvaria from Apert fetuses with the most frequent Ser252Trp FGFR-2 mutation (*a*, *c*, and *d*) compared with age-matched normal fetal calvaria (*b* and *e*). Apert fetal calvaria showed a marked increase in matrix (*M*) formation (*arrowheads*) beneath the periosteum (*P*) compared with normal age-matched normal fetus (*b*). High power microphotograph shows the abundant matrix (*M*) formed by subperiosteal cells below the periosteum in Apert fetus (*c*). The population of ALP-positive (*arrowheads*) subperiosteal cells, preosteoblastic cells forming new matrix (*M*), and osteoblasts along the calcified bone (*B*) was higher in Apert fetus (*d*) compared with age-matched fetal control (*e*). 5-μm-thick sections stained with von Goldner trichrome (*a* and *c*), von Kossa (*b*), or ALP (*d* and *e*); original magnification ×125 in *a*, *b*, *d*, and *e*; ×250 in *c*.

osteoblast maturation. We examined the histological consequences of the different FGFR-2 mutations on calvarial ossification in Apert fetuses. Histological analysis of fetal calvaria samples showed clear abnormalities in Apert fetuses. Histomorphometric analysis of the calcified bone matrix showed that the total amount of calcified bone matrix was much higher in Apert fetuses than in age-matched controls (not shown), consistent with premature ossification of the calvaria. The pattern of membranous osteogenesis in all Apert fetuses examined was characterized by increased de novo formation of bone matrix beneath the periosteum. Extensive matrix formation was extending along the subperiosteal area in Apert fetal calvaria (Fig. 1 *a*), whereas in normal age-matched calvaria, new matrix formation was restricted to a small fraction of the subperiosteal surface (Fig. 1 *b*). The increased subperiosteal matrix formation in fetal Apert calvaria was clearly evident at high magnification (Fig. 1 *c*). The histochemical staining of Apert calvaria showed that subperiosteal cells stained strongly for ALP (Fig. 1 *d*), indicating that the cells forming the subperiosteal matrix were committed to the osteogenic phenotype. Preosteoblastic cells and osteoblasts present along the bone matrix also stained strongly for ALP (Fig. 1 *d*). ALP staining was stronger in subperiosteal and osteoblastic cells in Apert calvaria (Fig. 1 *d*) than in normal age-matched control calvaria (Fig. 1 *e*), suggesting increased maturation of uncommitted precursor cells in Apert calvaria. The histomorphometric analysis showed that the subperiosteal bone surface with ALP-positive cells and osteoid was higher in calvaria from Apert fetuses than in control fetuses (96.3±1.6%, $n = 4$, vs. 66.8±4.1%, $n = 4$, $P < 0.001$), indicating that the increased maturation of preosteoblastic cells led to increased new matrix formation in Apert syndrome. In contrast, the osteoid and osteoblast surfaces were nearly normal at a distance from the periosteum (data not shown), suggesting that the main abnormality in Apert fetuses was restricted to the subperiosteal area. These histological results indicate that the premature calvaria ossification in Apert syndrome is associated with increased maturation of preosteoblastic cells leading to increased matrix formation at the subperiosteal area during fetal development.

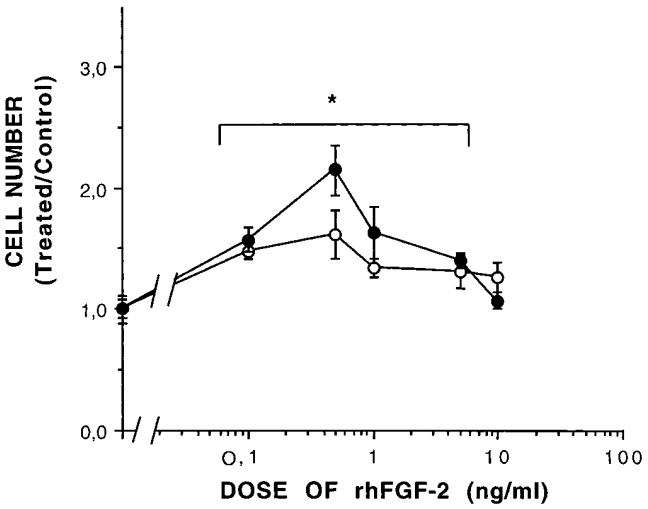

Figure 3. Normal response to rhFGF-2 in Apert calvaria cells. Treatment with rhFGF-2 for 48 h in serum-free medium induced a similar biphasic effect on cell proliferation in fetal calvaria cells with the Ser252Trp mutation (●, Ap cells) and fetal control cells (○, Co cells). Data are the mean±SEM of three to four cultures (*$P < 0.05$ vs. untreated cells).

Calvaria cells from Apert patients show normal cell growth. We then analyzed whether FGFR-2 mutations in Apert patients induced alteration of cell growth in calvaria cells. Time course analysis of DNA synthesis during 14 d of culture showed no abnormality in proliferation induced by Apert mutations in the population of calvaria cells studied and cultured in basal culture conditions. Fig. 2 *A* shows that the maximal DNA synthesis, evaluated by [^{3}H]thymidine incorporation into DNA, in calvaria cells from Apert infants and fetuses was not

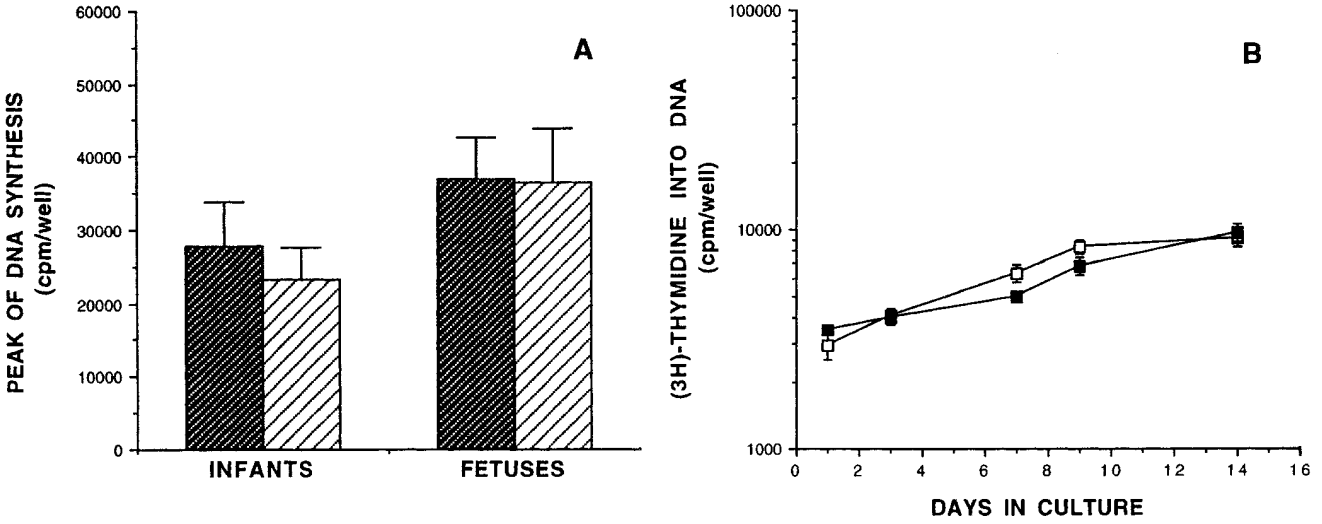

Figure 2. Calvaria cells from infants and fetuses with Apert syndrome showed normal cell growth. The mean maximal DNA synthesis in calvaria cells was similar in Apert infants and fetuses (*black bars*) compared with age-matched controls (*white bars*) (*A*). The time course analysis (*B*) showed that DNA synthesis was similar in fetal calvaria cells with the Ser252Trp mutation (■, Ap cells) and in normal fetal cells (□, Co cells). Data are the mean±SEM of three to four cultures.

different from age-matched control cells. Even in long-term culture (14 d), the cumulative DNA synthesis in calvaria cells did not differ in Apert infants or fetuses compared with age-matched controls (data not shown). Fig. 2 *B* shows that the time course of DNA synthesis was similar in fetal calvaria cells with the Ser252Trp mutation (Ap cells) and in normal fetal cells (Co cells). These data indicate that FGFR-2 mutations in Apert infants and fetuses do not increase proliferation in calvaria cells at the stage of maturation present in this in vitro system.

We then examined the response to exogenous FGF-2 in calvaria Ap cells with the Ser252Trp mutation. Fig. 3 shows the dose–response effect of rhFGF-2 on cell number in Ap and Co cells after 48 h of treatment in serum-free medium. rhFGF-2 induced a biphasic effect on cell proliferation in Ap and Co cells with a maximal stimulation at 0.5 ng/ml. The maximal stimulatory effect of rhFGF-2 on DNA synthesis did not differ significantly in Ap (+214%) and Co cells (+161%). This was confirmed by measuring DNA synthesis using [^{3}H]thymidine incorporation into DNA (maximal increase at 0.5 ng/ml rhFGF-2: +193% vs. +150% in Ap vs. Co cells, NS). These findings indicate that Ap fetal cells with the FGFR-2 Ser252Trp mutation are stimulated to proliferate in a similar fashion to control cells.

Apert FGFR-2 mutations lead to increased differentiation markers in calvaria cells. We then examined the expression of differentiation markers in calvaria cells from Apert subjects. Fig. 4 *A* shows that the mean ALP activity was higher (+56%) in calvaria cells from Apert infants compared with age-matched control cells. In fetal cells with the Ser252Trp mutation, the basal ALP activity was also higher (+201%) than the mean ALP activity in normal fetuses (Fig. 4 *A*). The basal levels of ALP activity were 6- and 10-fold higher in Ap fetal cells than in Co fetal cells at 48 and 96 h of culture, respectively (Fig. 4 *B*). In these mutant cells, the basal ALP activity remained elevated at all successive passages (fourth to eighth, by four- to sixfold, $P < 0.001$), documenting the permanent elevation of ALP expression. The elevated basal ALP activity in Ap cells was not modified by rhFGF-2 at doses ranging from 0.1 to 10 ng/ml (data not shown). On the other hand, cytochemical

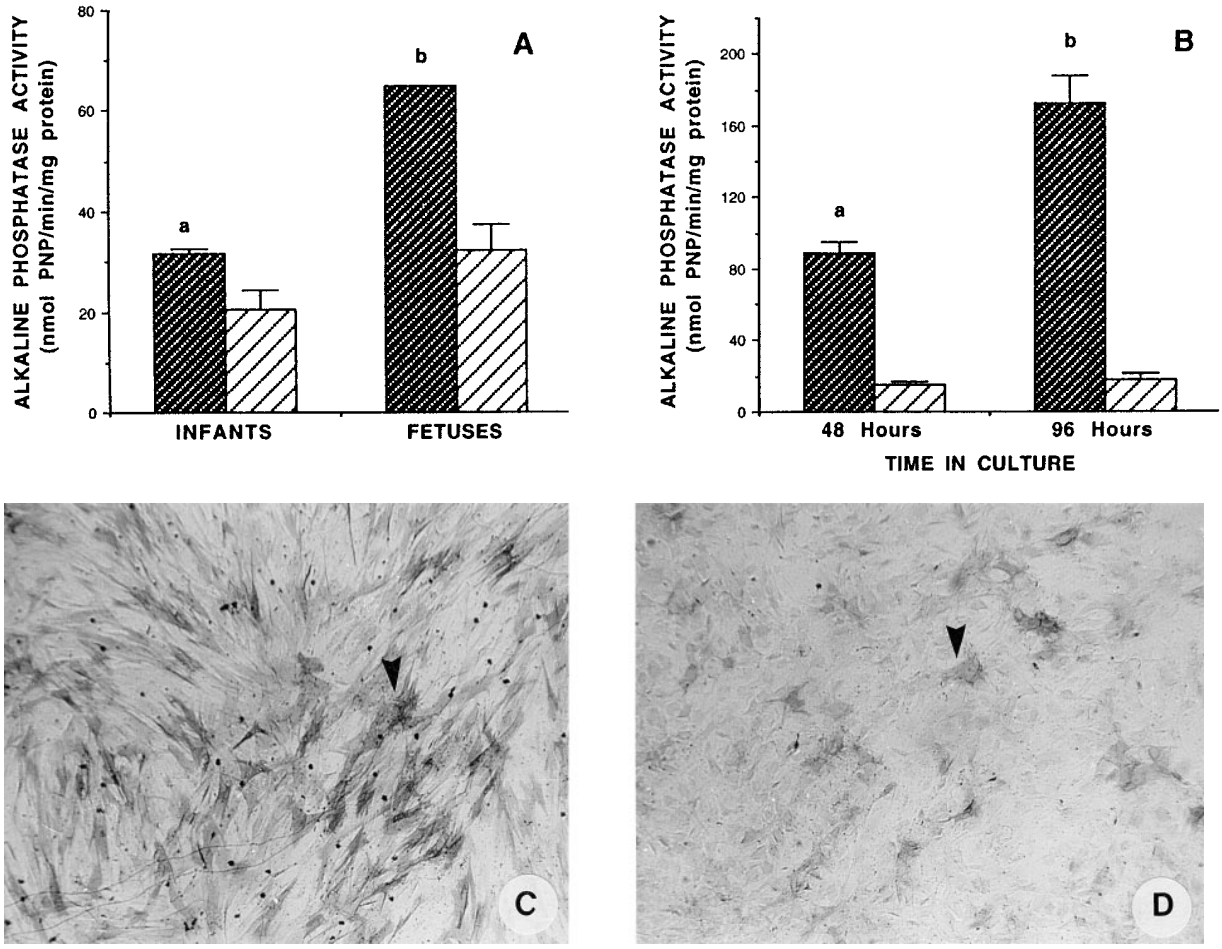

Figure 4. Increased basal ALP activity, expressed as nanomoles of *p*-nitrophenol (*PNP*) released per minute per milligram of protein, in Apert calvaria cells. The basal ALP activity in calvaria cells from Apert infants and fetuses (*black bars*) was increased compared with normal age-matched controls (*white bars*) (*A*). ALP activity in fetal calvaria cells with the Ser252Trp mutation (*black bars*, Ap cells) was higher than in control fetal cells (*white bars*, Co cells) at 48 and 96 h of culture in serum-free medium (*B*). Data are the mean±SEM of three to four cultures (*a* and *b*, $P < 0.05$ vs. the respective control cells). Cytochemical analysis showed that the number of ALP-positive cells (*arrowheads*) was much higher in Ap calvaria cells with the Ser252Trp mutation (*C*) compared with Co cells (*D*) (original magnification ×125).

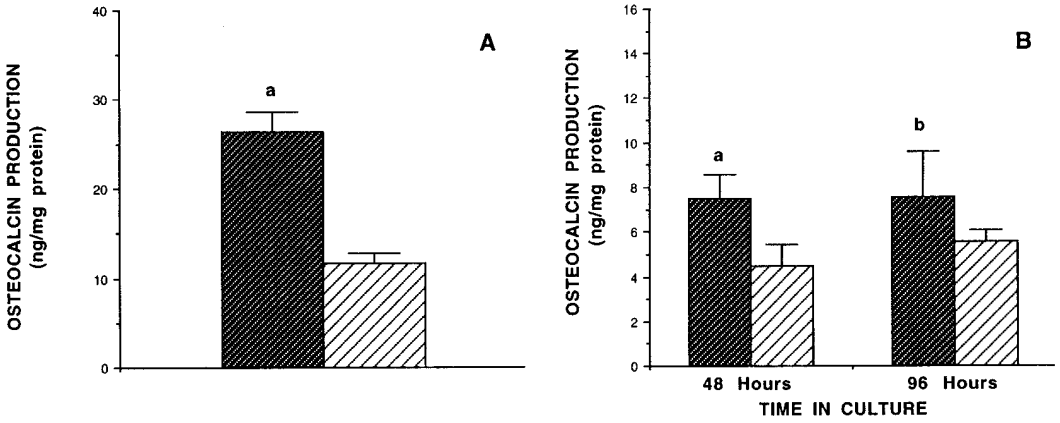

Figure 5. Increased OC production by Apert calvaria cells. OC production by calvaria cells from Apert infants (*black bars*) was higher than in age-matched control calvaria cells (*white bars*) (*A*). OC production was also higher in fetal calvaria cells with the Ser252Trp mutation (*black bars*, Ap cells) than in control fetal cells (*white bars*, Co cells) at 48 and 96 h of culture in serum-free medium (*B*). Data are the mean±SEM of three to four cultures (*a* and *b*, $P < 0.05$ vs. the respective control cells).

analysis showed that ALP-positive Ap calvaria cells (Fig. 4 *C*) were more numerous than ALP-positive Co cells (Fig. 4 *D*). This was confirmed by quantification of the percentage of ALP-positive cells. Most Ap cells were ALP-positive, and the percentage of ALP-positive cells was about fourfold greater than in Co cells (80.5±1.3 vs. 24.2±1.9% of total cells, $P < 0.001$). The increased number of ALP-positive cells suggests that the Ser252Trp mutation led to an increased commitment of immature calvaria cells to the osteoblastic phenotype.

Analysis of osteocalcin production showed that OC levels were 2.3-fold higher in calvaria cells from Apert infants compared with age-matched control cells (Fig. 5 *A*). In Ap fetal calvaria cells with the Ser252Trp mutation, OC production was 66 and 36% higher at 48 and 96 h of culture, respectively, than in Co cells (Fig. 5 *B*). In addition, the basal Col 1 production was twofold higher in Ap cells compared with Co cells, as evaluated by P1CP levels in the medium (50.2±3.3 vs. 24.5±3.0 μg/mg protein, $P < 0.01$). Treatment with rhFGF-2 (0.1–10 ng/ml) did not affect P1CP release, which remained elevated in Ap cell cultures (data not shown). To confirm these data, we examined the expression of ALP and Col 1 genes in immortalized fetal Ap cells and Co cells cultured in basal conditions. Fig. 6 shows that the basal mRNA levels for ALP and Col 1 were increased in imAp cells compared with imCo cells. These results indicate that FGFR-2 mutations resulted in increased expression of osteoblast differentiation markers in Apert calvaria cells, and that this increased expression is independent of exogenous FGF-2 stimulation.

Ser252Trp mutation in calvaria cells leads to increased matrix production in culture. To investigate further the altered phenotype induced by the Ser252Trp mutation in fetal calvaria cells, we examined the production of matrix by imAp and imCo cells cultured in aggregates (32). Cytochemical analysis of the aggregates formed after 2 wk showed that only a fraction of imCo cells stained for ALP in aggregates, whereas most aggregated imAp cells were strongly ALP-positive (data not shown), confirming that the Ser252Trp mutation increased the population of committed preosteoblastic cells. Histological analysis of the aggregates at 2 wk showed that imAp cells formed an abundant matrix (Fig. 7 *a*). Most of the abundant matrix formed was mineralized, as revealed by von Kossa staining (Fig. 7 *c*). In contrast, imCo cells cultured under identical conditions formed a small amount of matrix (Fig. 7 *b*), and the amount of mineralized matrix was lower in imCo cells than in imAp cells (Fig. 7 *d*). These data indicate that the Ser252Trp mutation in immortalized fetal Ap cells induces an increased formation of mineralized matrix in culture. Taken together, the in vivo and in vitro data indicate that FGFR-2 mutations in Apert fetuses increase the number of uncommitted precursor cells that enter the osteogenic pathway, leading ultimately to increased matrix formation and premature calvaria ossification.

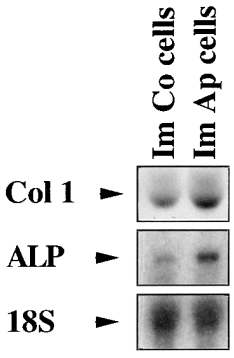

Figure 6. Northern blot analysis showing increased basal Col 1 and ALP gene expression in immortalized Apert fetal calvaria cells with the Ser252Trp mutation (*imAp*) compared with imCo fetal cells. Total cellular RNA (20 μg) was subjected to Northern blot analysis. The membrane was hybridized with ^{32}P-labeled ALP and Col 1 human cDNA probes, then stripped and rehybridized with an 18S probe.

Discussion

Although several mutations in FGFR-2 have been found to cause craniosynostosis syndromes, the effects of these mutations on human calvaria cell phenotype have not been identified. In this study, we analyzed the phenotypic consequences of FGFR-2 mutations on the proliferation and differentiation of calvaria cells in Apert syndrome. The data show that FGFR-2 mutations in this syndrome increase the maturation of preosteoblastic calvaria cells, leading ultimately to increased matrix formation and premature calvaria ossification.

Although most of our Apert patients showed a Ser252Trp mutation, other mutations were found in exon IIIa of FGFR-2.

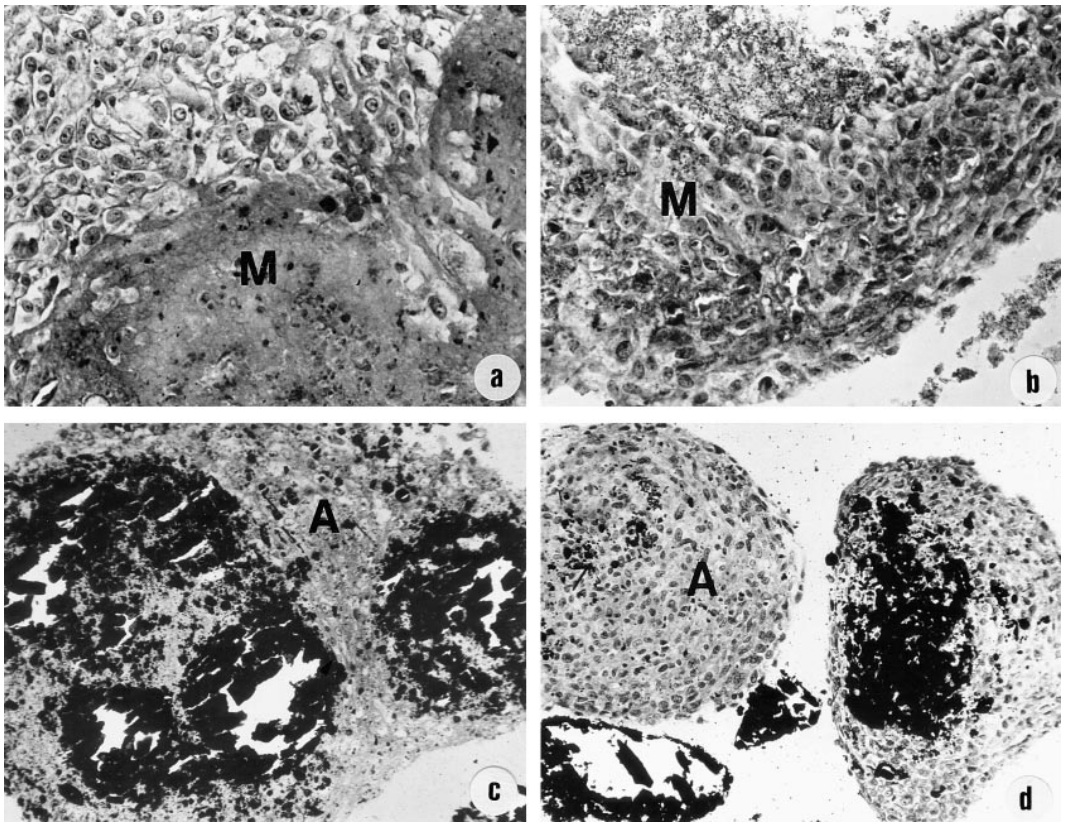

Figure 7. Histological analysis showing that the Ser252Trp mutation in FGFR-2 leads to an increased matrix production in culture. Immortalized fetal Ap and Co calvaria cells were cultured in aggregates for 2 wk in the presence of ascorbic acid and phosphate. The fixed cultures were embedded, and 5-μm-thick sections were stained with Goldner trichrome (*a* and *b*) or von Kossa counterstained with toluidine blue (*c* and *d*). In all cultures examined, imAp cells (*a*) formed an increased amount of matrix (*M*) compared with imCo cells (*b*). The amount of mineralized matrix formed by imAp cells was also greater (*c*) than in imCo cells (*d*) cultured in aggregates (*A*) in the same conditions (original magnification ×250).

These mutations were also localized in the linker domain between IgII and IgIII, and may thus induce similar alteration of the cell phenotype. Indeed, we found that all Apert fetuses showed the same histological pattern, characterized by increased formation of subperiosteal matrix and premature calvaria ossification compared with age-matched fetuses. The possible mechanisms by which FGF-2 may induce skeletogenesis include increased cell mitogenesis (15–17) and promotion of cell differentiation (20–23). Thus, we investigated the effects of FGFR-2 mutations on calvaria cell proliferation and differentiation in Apert patients. We found that the basal growth of preosteoblastic calvaria cells was not increased in either Apert infants or fetuses, suggesting that FGFR-2 mutations in this syndrome do not result in increased proliferation of these cells. Although FGFR-2 mutations may affect the growth of uncommitted osteoprogenitor cells present in the marrow stroma, the proliferative rate of these cells was not determined in our culture system, and it is unknown whether the mutations affected the proliferation of this cell population. In addition to basal cell growth, we found that the proliferative response to rhFGF-2 was not altered in mutant fetal calvaria cells. This may result from rhFGF-2 binding to FGFR-1 homodimers or to FGFR-1/FGFR-2 normal heterodimers, since FGF-2 can bind to different FGFRs (34, 35) and thereby initiate transcriptional activation signals (36). Alternatively, exogenous FGF-2 may also be translocated with FGFR-1 into the nucleus and activate nuclear effectors and gene transcription (37, 38).

In contrast to cell growth, the cytochemical, biochemical, and molecular analyses of osteoblast markers indicate that the osteoblast maturation pathway is altered in Apert calvaria cells. We found that ALP, OC, and Col 1 expression was increased in mutant calvaria cells, and the increase in differentiation markers was not influenced by exogenous FGF-2 stimulation, indicating that the FGFR-2 mutations led to increased osteoblastic cell maturation independently of ligand binding. Our finding that the proportion of ALP-positive cells was much higher in Apert cells compared with control cells is in accordance with the increased ALP expression in subperiosteal cells on histological sections, and suggests that the mutation led to an increase in the number of uncommitted precursor cells that entered the osteogenic pathway. Calvaria ALP-positive cells are believed to be putative osteoprogenitor cells in vitro (39). Accordingly, we found that the increased pool of ALP-positive cells was associated with an increased extent of matrix formed by subperiosteal ALP-positive cells in Apert calvaria, and with an increased amount of mineralized matrix in vitro. These in vivo and in vitro data indicate that FGFR-2 mutations in Apert syndrome increase the maturation rate of preosteoblastic cells, leading ultimately to increased mineralized matrix formation and premature calvaria ossification. These results establish for the first time a connection between the altered genotype and cellular phenotype induced by FGFR-2 mutations in Apert syndromic craniosynostosis.

Mutations in FGFRs have been reported to affect ligand binding and receptor signaling (9–12). Structural analysis and transfection experiments indicate that mutations in FGFR-2 associated with Crouzon syndrome (11, 12), or mutations in FGFR-3 associated with achondroplasia and thanatophoric dysplasia (10, 40, 41) induce ligand-independent receptor activation. However, the constitutive activation of FGFRs induced by these mutations is associated with variable phenotypic effects on cell growth (10, 41, 42) and differentiation (11, 40), de-

pending on the cell type or FGFR mutation. This study indicates that FGFR-2 mutations in Apert syndrome affect cell maturation in human calvaria preosteoblastic cells. Using this model, identification of the receptor signaling mechanisms implicated in the increased differentiation pathway can now be undertaken to understand the cascade of events leading to the premature calvaria ossification in Apert syndrome.

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