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Role of the extracellular matrix and growth factors in skull morphogenesis and in the pathogenesis of craniosynostosis

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ABSTRACT The complex and largely obscure regulatory processes that underlie ossification and fusion of the sutures during skull morphogenesis are dependent on the conditions of the extracellular microenvironment. The concept that growth factors are involved in the pathophysiology of craniosynostosis due to premature fusion of skull bone sutures, is supported by recent genetic data. Crouzon and Apert syndromes, for example, are characterized by point mutations in the extracellular or transmembrane domains of fibroblast growth factor-2 receptor. In primary cultures of periosteal fibroblasts and osteoblasts obtained from Apert and Crouzon patients, we observed that Crouzon and Apert cells behaved differently with respect to normal cells as regards the expression of cytokines and extracellular matrix (ECM) macromolecule accumulation. Further modulation of ECM components observed after the addition of cytokines provides support for an autocrine involvement of these cytokines in Crouzon and Apert phenotype. Changes in ECM composition could explain the altered osteogenic process and account for pathological variations in cranial development. We suggest that a correlation exists between *in vitro* phenotype, clinical features and genotype in the two craniosynostotic syndromes. New research into signal transduction pathways should establish further connections between the mutated genotype and the molecular biology of the cellular phenotype.

KEY WORDS: *skull morphogenesis, craniosynostosis, Crouzon and Apert syndromes, growth factors, extracellular matrix.*

Introduction

The development of cranial primordia involves fundamental processes characterized by growth, morphogenesis, cell differentiation and pattern formation. Interactions between extracellular matrix (ECM) components, growth factors and embryonic tissues induce cell differentiation pathways and inductive events. During skull development, osteogenetic events are characterized by continuous deposition of osteoid matrix and by resorption of the calcified bone matrix due to the changing curvature of the bones during development (Sullivan, 1986). The most severe anomalies of the calvarium, such as Crouzon and Apert syndromes, are characterized by premature fusion of skull sutures due to altered osteogenic processes at the time of calvarian development. It is possible that persistence and premature ossification of fetal sutures initiate synostosis before the sutures become fully developed. Biochemical and molecular aspects of craniofacial development, such as the biological regulation of normal or premature cranial suture fusion, are still largely unknown. In this review we summarize the evidence that supports the hypothesis that the genetic control of skull development is mediated by the regulation of matrix components and interactions between cytokines and cell surface receptors.

Cranial articulation pattern and ossification processes during development

Embryological and histological observations of human fetal and embryonic specimens have revealed the outlines of the developmental history of prenatal skull articular system, which plays a great functional role during skull morphogenesis (Pitchard *et al.*,

Abbreviations used in this paper: Extracellular matrix (ECM), basic fibroblast growth factor or fibroblast growth factor-2 (FGF 2), fibroblast growth factor receptors (FGF Rs), transforming growth factor β (TGFβ), interleukins (ILs), glycosaminoglycan (GAG), proteoglycan (PG), chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronic acid (HA), heparin sulfate (HS), fetal calf serum (FCS), Eagle's minimum essential medium (MEM).

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1956, Morris-Kay and Tucket, 1991). Eight-week old human embryos present articulations between frontal, sphenoid and ethmoid bones defining a coronal ring (Venes and Burdi, 1985). At this time, the midline articulation between the sphenoid and etmoid bones, which is properly named the sphenoethmoidal synchondrosis (Burdi *et al.*, 1986), is cartilaginous and the portions of the ring separating the frontal and sphenoid bones show the typical histology of sutures. At all stages from their earliest development up to and including adult stages, the sutures present five distinct layers passing from one bone to the edge of the other. These layers are composed of the first cambial layer, the first fibrous capsule, the loose cellular middle zone, the second fibrous capsule and the second cambial layer.

The morphogenetic organization of sutures differs between the face and the vault. In face sutures, cambial and capsular layers are present before the suture is formed, and the middle and uniting layers are derived from mesenchyme between bones. In the cranium vault, capsular layers are formed only after the cambial layers have almost met, and the uniting and the middle layers are derived from the delamination of the fibrous ectomeninx between the bones (Pitchard *et al.*, 1956). During skull development, the cambial layer is gradually reduced to a single layer of flattened osteoblasts, the capsular layer appears to thicken and the middle layer becomes increasing vascular. The uniting layers form a bond of union between adjacent bones, permitting slight movements and marginal expansion during the skull's period of growth. The ossification sequence during prenatal development of the human cranium has been identified (Kiajer, 1990). At 6-weeks of development, mesenchymal condensations prefigure the bone of basicranium, and in the 7th week chondrification of the basicranium begins (Delezoide *et al.*, 1998). Ossification and mineralization of the cranial vault take place directly in the mesodermic membranes, beginning from different ossification centres during the 8th week of gestation. In the mesenchymal membrane, proliferative pre-osteoblasts differentiate into osteoblasts, and deposition of unmineralized bone matrix starts in six ossification centres (two frontal, two parietal and two occipital). At 18-weeks, the mineralizing bones meet along their edges where sutures are formed.

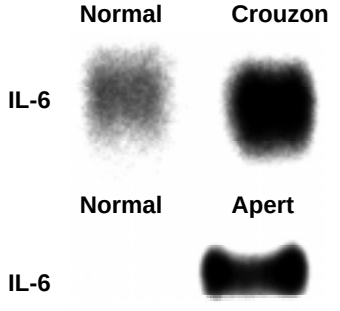

Fig. 1. Increased expression of IL-6 mRNA in Crouzon and Apert fibroblasts. *Total cell RNA was extracted from Crouzon and Apert fibroblasts and from normal fibroblasts used as controls, which had been seeded at the density of 6x10^5 cells/flask and maintained for 4 h in MEM + 10% FCS. For Northern hybridisation technique, 15 μg of total RNA were electrophoresed on 1% agarose gels containing formaldehyde, blotted onto nitrocellulose filters and hybridised with a ^{32}P-labelled IL-6 cDNA probe. (From Bodo et al., 1996, and Bodo et al., 1998).*

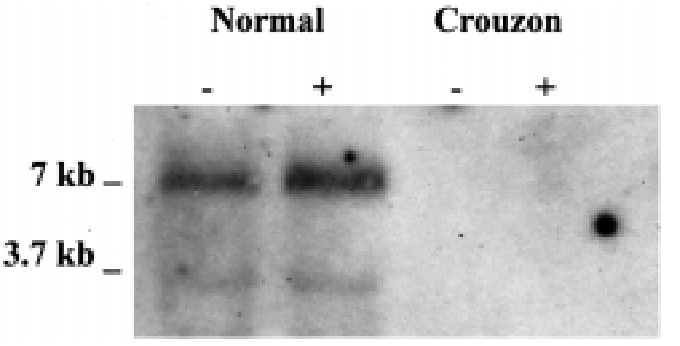

Fig. 2. Expression of FGF 2 mRNA in human normal and Crouzon osteoblasts in the presence (+) or absence (-) of FGF 2. *Total cell RNA was extracted from normal and Crouzon osteoblasts, which had been seeded at a density of 2x10^6 cells/flask and maintained for 24 h in MEM + 0.5% FCS with or without FGF 2 (20 ng/ml). For Northern hybridisation, 20 μg of total RNA were electrophoresed in 1% agarose gels containing formaldehyde, blotted onto nitrocellulose filters and hybridised with a ^{32}P-labelled FGF 2 cDNA probe. (From M. Bodo et al., 1999).*

Sutures contain osteogenic stem cells and periosteal fibroblasts that differentiate into osteoblasts capable of producing new bone tissue, and are thus considered active sites of bone growth. Cranium grows by apposition of pre-mineralized osseous matrix along the edge of the sutures. The end result of ossification of cranial vault sutures is the disappearance of the osteogenic area interposed between the partially overlaid bone margins. The premature fusion of one or more sutures prevents further bone growth along the edges. This leads the cranial vault to expand in other directions, thereby delaying the fusion of other sutures and giving rise to a wide variety of pathological phenotypes.

Morphogenetic signals of ECM components during craniofacial development

During osteogenesis, mesenchymal cells differentiate in osteoblast lineage and produce a mineralized ECM that takes control of morphogenic events (Morris-Kay and Tucket, 1991). The ECM complex is formed by proteoglycans (PG), glycosaminoglycans (GAG), fibronectin, collagens, and other glycoproteins, which are differently distributed and organised in tissues and stages of development. It has been suggested a model of "dynamic reciprocity" between the ECM and cellular components such as the cytoskeleton (Giancotti and Ruoslahti, 1999). The ECM-cell-receptor-link transmits signals across the cell membrane in the cytoplasm, thereby initiating a cascade of events that culminates in the expression of specific genes (Adams and Watt, 1993). Much experimental evidence demonstrates that osteogenetic processes fall largely under the balanced control of interactions between cells and the ECM (Martins-Green and Bissel, 1995). Fibronectin, for example, is distributed in areas of skeletogenesis and controls the early stages of bone formation regulating the recruitment and commitment of osteoblasts to terminal differentiation (Gronowicz *et al.*, 1991). Osteoblast-fibronectin interactions are required for bone morphogenesis, since fibronectin regulates the matrix assembly and interacts with type I collagen fibrils (Nordahl *et al.*, 1995, Moursi *et al.*, 1996). Collagen is another ECM molecule that provides a morphogenetic signal. It realizes a matrix-mediated tissue interaction fundamental for embryo chondrocranial morphogenesis (Wood *et al.*, 1991). Laminin, fibronectin, collagen type I

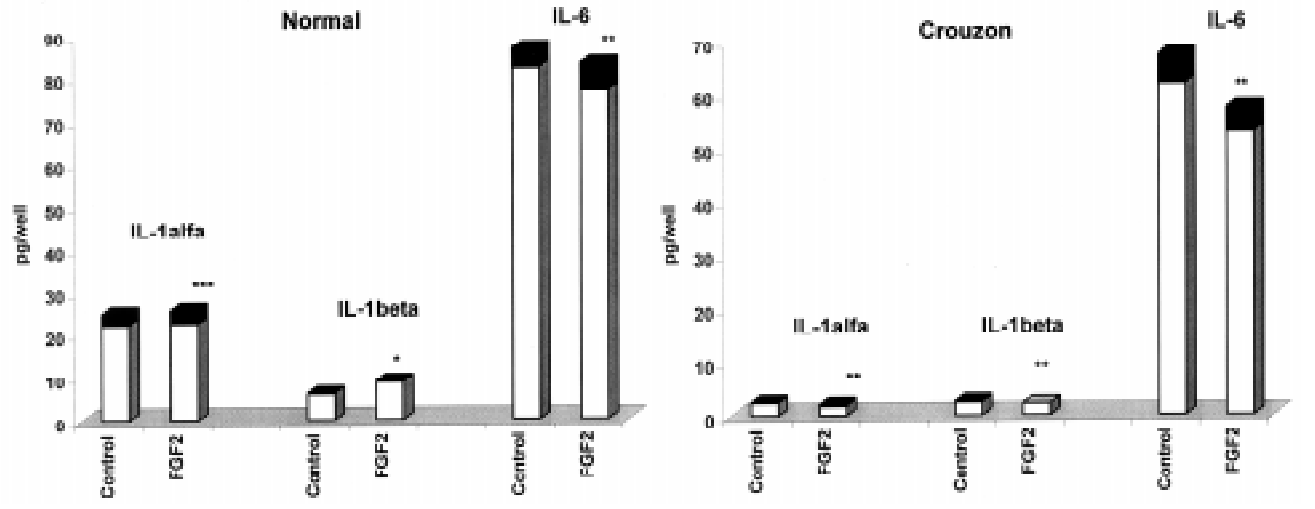

Fig. 3. IL-1α, IL-1β and IL-6 levels in conditioned media obtained from normal and Crouzon osteoblasts with or without FGF 2 (20 ng/well). *Cells were maintained for 24 h in MEM + 0.5% FCS with or without FGF 2 (20 ng/ml) followed by 24 h in serum-and growth factor-free medium. Supernatants were harvested and the levels of IL-1α, IL-1β and IL-6 were measured using ELISA kits. Each value represents the mean ± SD of 8 measurements. Significance versus each control: P<0.001*; P<0.01**; not significant***. (From Bodo et al., 1999)*

and IV are all distributed in characteristic maps in epithelial-mesenchymal interfaces involved in the formation of avian embryo cartilaginous neurocranium (Thorogood *et al.*, 1986, Thorogood, 1987, 1988). Type I and II collagens are differently localised in rat sphenoccipital synchondrosis and in the intermaxillary suture (Mizoguchi *et al.*, 1992), and the differences in the expression of collagen types could explain the different modes of chondrogenesis in the two craniofacial regions.

Variations in the distribution of collagen I and III were also observed in developing human calvarial bones. Pro-alpha (1) collagen mRNA is expressed in osteoblasts, fibroblasts of periosteum, *dura mater,* at or near the upper surface of calvarial bones, indicating the direction of bone growth (Sandberg *et al.*, 1988). Type III collagen, called "fetal collagen", because of its abundance in fetal tissues, has been detected only in condensed mesenchyme between the bone spikules and at lower levels in the fibroblasts of periosteum (Van der Mark, 1981). The patterns of expression of these two types of collagen are independent and are not coordinated in calvarial cells during the stages of craniofacial development.

The complex progression of chondrocyte differentiation during endochondral ossification is also controlled spatially and temporally by other ECM components such as PG, and particularly by syndecan-III and biglycan (Shimazu *et al.*, 1996, Xu *et al.*, 1998). The finding that heparan sulphate GAG chains are also involved in chondrocyte proliferation (Chintala *et al.*, 1995), is in keeping with the hypothesis that many ECM components regulate ossification during fetal development and play a role in the terminal differentiation of embryonic cells. In addition, an important role in the craniofacial morphogenesis is ascribed to matrix metalloproteinases, enzymes involved in ECM degradation. For example, the expression of gelatinase in many areas of the craniofacial complex in the developing mouse indicates that this enzyme plays a significant role in localised tissue remodelling during craniofacial morphogenesis (Iamaroon *et al.*, 1996).

All these different transient patterns of ECM components are interpreted as reflecting different levels of morphogenetic specification of skull form in the developing head.

Role of growth factors in skull morphogenesis and osteogenesis

A significant role in skull morphogenetic events is played by signal molecules present in the early embryo *in vivo*. Transforming growth factor β (TGFβ), interleukins (ILs) and basic fibroblast growth factor (bFGF or FGF 2) are putative "signal peptides" present in skull tissues at the time of active differentiation and morphogenesis. TGFβ regulates a variety of osteoblast activities related to bone formation. It enhances collagen synthesis and matrix apposition (Centrella *et al.*, 1994, Yamaguchi, 1995), modulates the expression of the chondrogenic phenotype (Frenz *et al.*, 1994) and controls ECM expression in human cleft palate cells *in vitro* (Bodo *et al.*, 1999a). Injection of TGFβ in the periosteal region stimulates membranous and endochondral bone formation *in vivo* (Joyce *et al.*, 1990, Rosen *et al.,* 1990). Many investigators have suggested a role for TGFβ isoforms in the biology underlying premature suture closure, via paracrine effects. Roth *et al.* (1997b) have shown that completely fused synostosed cranial sutures display immunoreactivity for TGFβ_1, β_2, β_3 isoforms, whereas TGFβ_3 expresses a different pattern at the margin of the patent sutures. Utilising an *in vitro* rat model, the same authors have also hypothesised that TGFβ_3 acts as an inhibitor of fusion in human sutures (Roth *et al.*, 1997a). The finding that *dura mater* is also actively able to produce TGFβ isoforms, suggests that they may have a paracrine signalling role in the regulation of suture fusion (Roth *et al.*, 1996). An *in vitro* rat coronal suture model has recently been utilised to demonstrate the capacity of osteoprogenitor cells of the cranial sutures to divide and synthesise ECM, and it has been suggested that *dura mater*-derived growth factors affect these processes and regulate the fusion or the patency of sutures (Oppermann *et al.*, 1996, 1997, 1998).

Interleukin 1 (IL-1) and interleukin 6 (IL-6) are two of other cytokines that are involved in the regulation of bone cell functions (Mundy and Bratter, 1995). IL-1 stimulates cell replication and, at a low dose, bone collagen synthesis (Canalis *et al.*, 1989); IL-6 enhances bone turnover, stimulating bone resorption processes (Ishimi *et al.*, 1990). Low IL-6 expression has been observed with rat posterior frontal suture fusion (Most *et al.*, 1998). This finding

has elicited contrasting interpretations: either the role of IL-6 in suture fusion is extremely potent with only a very low amount of transcript being required to translate proteins, or IL-6 has a minor role in frontal suture fusion.

FGF 2, which is the most abundant growth factor in the vault, is another candidate for regulation of bone development (Hurley and Forkiewicz, 1996). It displays a broad spectrum of activities, including growth increase induction of proteases such as plasminogen activator, catepsin B, kallicrein and increase of cell migration (Szebenyi and Fallon, 1999). The FGF family members are able to bind four different transmembrane tyrosine kinase receptors (FGF Rs 1-4) (McWhirter *et al.*, 1997). Genetic analysis of many human skeletal disorders have demonstrated the critical role of the FGF-FGF R system in endochondral and endomembranous ossification. In a recent study, Delezoide *et al.* (1998) reported differential expression of FGF R 1-3 genes during head ossification processes in the human embryo. At 8 weeks, FGF R 3 genes are markedly expressed in the developing skull, in particular in the pre-osteoblasts prior to osteoblastic secretion of osteoid. Strong expression of FGF R 1-3 is detected in facial endomembranous bones. Expression of the FGF R 1 and 2 genes appears in the ossification centres of sutures, and FGF R 2 transcripts are localised in the external fibrous periosteum. The authors suggested that the distinct spatial and temporal expression patterns of FGF R 1-3 genes at different development stages of endochondral and membranous bones, are involved in signalling pathways controlling osteogenesis during development. Interactions of FGFs with FGF Rs of progenitor stem cells are in fact critical for their differentiation into osteoblasts (Iseki *et al.*, 1997).

Pathogenesis of craniosynostosis and recent findings obtained in an in vitro model

The numerous clinical phenotypes of craniosynostosis syndromes due to premature fusion of skull sutures, include abnormal head shape, protruding eyes and midface underdevelopment. The syndromes show different patterns of limb involvement. Normal hands and feet are present in Crouzon syndrome, broadened thumbs and toes in Pfeiffer syndrome, severe fusion of the bones of the hands and feet in Apert syndrome (Cohen, 1986; Cohen, 1993; Cohen and Kreiborg, 1993; Gorlin *et al.,* 1990). Comparative studies indicate that although Apert and Crouzon syndromes (Carinci *et al.,* 1994), present very similar cranial anomalies, they differ in cranial development (Kreiborg *et al.*, 1993). In Apert syndrome, the primary abnormality appears to involve the cartilage of the anterior cranial base, in particular the wings of the sphenoid and the ethmoid bones. Apert necropsy specimens show patent spheno-occipital syncondrosis with disorganised cartilage cells that could be the cause of the abnormal growth and ossification. The infant Apert calvaria are characterized by premature fusion of the coronal sutures, while all other sutures and fontanelles are patent. In contrast, Crouzon calvaria are characterized by more complex synostoses involving the coronal sutures and fontanelles. A recent study showed a significantly more occipital localisation of the frontal bone centre and a more frontal localisation of the parietal bone centre at the side of a synostotic coronal suture only in Apert calvaria (Mathijssen *et al.*, 1996). The finding that these bone centre localisations are not present in Crouzon syndrome, demonstrates that the two syndromes have a different pathogenesis. Recent findings from clinical molecular genetic studies identified a mutation in homeotic gene MSX2 as the first genetic defect in craniosynostosis type 2, also known as Boston type, (Muller *et al.,* 1997). In Crouzon, Pfeiffer, Jackson-Weiss and Apert syndromes, which are more common with respect to the Boston type, the mutations were identified in the gene coding for FGF receptor 2 (FGF R 2) and located in the domain important for binding (Reardon *et al.*, 1994, Wilkie *et al.*, 1995a,b, Wilkie and Wall, 1996, Wilkie, 1997). In particular, the mutations were identified in exons 5 and 7 of the gene which codes for immunoglobulin (Ig)-like chain III and in the region of the receptor linking IgII and IgIII (Galvin *et al.*, 1996, Muller *et al.*, 1997). In some cases of Crouzon and Pfeiffer syndromes, less frequent mutations were observed in FGF R 1 and FGF R 3 (Muller *et al.*, 1997). It is surprising that the same gene defect induces craniosynostosis syndromes with a wide spectrum of craniofacial abnormalities and highly variable phenotypes (Park *et al.*, 1995, Rutland *et al.*, 1995, Pulleyn *et al.*, 1996, Slaney *et al.*, 1996). During the last few years, a growing number of point mutations have been mapped in all three FGF receptors and have been involved in skeletal and cranial development disorders (Bellus *et al.*, 1996, Neilson and Freisel, 1995), but many questions still remain unanswered. Since FGF Rs have distinct patterns of expression during embryogenesis, it is reasonable to hypothesise that each receptor mediates different developmental responses to FGFs. Contrasting data have been reported on the implications of FGF R activity in suture fusion or in their patency (Bresnick and Schendel, 1995, Neilson and Freisel, 1995). Further studies are therefore necessary to clarify the development consequences of mutated receptors activity. An interesting hypothesis is that the heterogeneity of phenotypes with similar mutations probably reflects involvement of other genes that modulate the effects of mutated FGF Rs (Webster and Donoghue, 1997). This concept calls in question the monogenic model for craniosynostosis syndromes (Mulvihill, 1995).

These intriguing questions prompted us to better clarify the pathophysiology of craniofacial malformations by studying, in an *in*

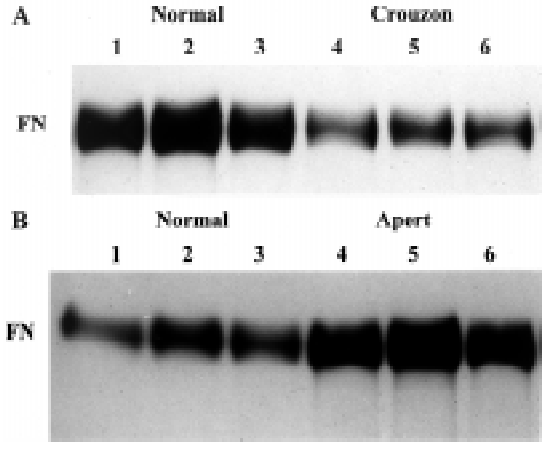

Fig. 4. Fibronectin (FN) secreted by Crouzon, Apert and normal fibroblasts, with or without IL-1 and IL-6. *Confluent cultures of normal and pathological fibroblasts were maintained in MEM + 2% synthetic serum containing no additives (lanes 1,4), 5U/ml IL-1 (lanes 2,5), or 5 U/ml IL-6 (lanes 3,6). After four hours, [^{35}S]-methionine (20μCi/ml, s.a.>1000Ci/mmol.) was added to cultures. Following a three hour labelling period, media were collected and subjected to gelatin-sepharose affinity chromatography. The isolated FN was analysed by electrophoresis on 6% SDS-polyacrylamide slab gels and fluorography. The figures show the film autoradiograms of the gels. The fluorographs were analysed and quantified by scanning densitometry. (From Bodo et al., 1996, 1997).*

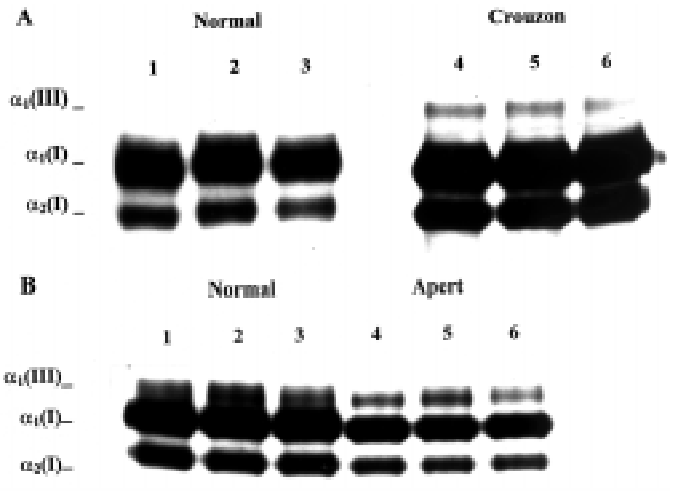

Fig. 5. Collagen polypeptides synthesised by Crouzon, Apert and control fibroblasts, treated with or without IL-1 or IL-6. *Confluent cultures of normal and pathological fibroblasts were maintained in MEM + 2% synthetic serum containing no additives (lanes 1,4), 5 U/ml IL-1 (lanes 2,5), or 5 U/ml IL-6 (lanes 3,6). After 24 h, [^{3}H]- proline (10 μCi/ml, s.a. 35 Ci/mmol.) was added to the cultures. Following a 24 h labelling period, aliquots of culture media containing [^{3}H]-labelled proline collagens were digested with pepsin at pH 2.2 for 15 h at 4°C. The pepsin-resistant collagens were denatured by boiling for 5 min and the constituent α chains were separated by SDS-PAGE on 60% cross-linked gels using delayed reduction. The figure shows the film autoradiogram of the gels. The migratory positions of the collagen α chain were determined from standards and their collagenous nature demonstrated by susceptibility to highly purified bacterial collagenase. (From Bodo et al., 1996, 1997).*

vitro model, the phenotype of cells obtained from Crouzon and Apert patients (Bodo *et al.,* 1996, 1997, 1998, 1999b). Cells were obtained from Apert and Crouzon patients (aged 1 and 18 years respectively), during corrective surgery for the malformations. Fibroblasts were obtained from the galea-pericranium and osteoblasts from specimen of the parietal bones. Control normal cells were established from explants removed from age-matched subjects being treated for parietal bone fractures.

Results

Expression of secreted cytokines

We analyzed mRNA expression of ILs, TGFβs and FGF 2 and their secretion in conditioned media (CM) obtained by Crouzon and Apert cells *in vitro*. These cytokines act together, via autocrine and/or paracrine mechanisms, to control the process of cranial morphogenesis. We found that while normal fibroblasts showed low levels of constitutive expression of IL6 mRNA, as analyzed by Northern blotting, higher amounts of IL6 transcript were detectable in both Crouzon and Apert fibroblasts (Bodo *et al.*, 1996, 1998) (Fig. 1), and the increased IL6 gene expression translated into a parallel increase of the secreted protein only in Crouzon fibroblasts. Quantification of IL1 levels and studies on its bioactivity, using a mouse thymocyte proliferation assay, showed that both IL1α and β were higher in Crouzon than in normal CM (Bodo *et al.*, 1996). In contrast, Apert CM presented significantly reduced amounts of both IL-1 isoforms (Bodo *et al.*, 1998). As regards TGFβ expression, while Apert fibroblasts CM showed no increase in the release of active $TGF\beta_1$ with respect to controls (Bodo *et al.*, 1998), Apert osteoblast CM displayed raised $TGF\beta_1$ expression. In particular, $TGF\beta_1$ mRNA expression was greater in Apert sphenoid osteoblasts as compared with Apert parietal osteoblasts (Locci *et al.,* 1999). No detectable amount of FGF 2 transcript was obtained from Crouzon osteoblasts even when treated with FGF 2 (Bodo *et al.*, 1999b, Fig. 2). However, studies still in progress, seem to show the presence of FGF 2 transcript in Crouzon fibroblasts up-regulated by FGF 2 treatment. Finally, our observations that FGF 2 caused a higher drop of IL-6 secretion in Crouzon than in control osteoblasts (Bodo *et al.*, 1999b, Fig. 3), and decreased the amount of TGFβ mRNA in Apert osteoblasts (Locci *et al.,* 1999), suggest that ILs, FGF 2 and $TGF\beta_1$ interact according to a cascade pattern. Since the interactions between the different cytokines and their feedback mechanisms appear critical in the regulation of osteoblast differentiation (Yamaguchi, 1995), we suggest that the different cytokine patterns observed in Apert and Crouzon cells with respect to normal ones may account for the pathological variations in cranial development.

Fibronectin synthesis

Secreted fibronectin was analyzed in media obtained from Crouzon and Apert periosteal fibroblasts labelled with ^{35}S-methionine. Fibronectin was isolated by affinity chromatography on gelatin-sepharose resin, subjected to SDS-PAGE analysis, followed by densitometric quantification of fluorographies. The amount of fibronectin produced by Crouzon fibroblasts was lower than that produced by normal fibroblasts (Bodo *et al.*, 1996, Fig. 4A), in contrast to that which occurs in Apert fibroblasts, in which fibronectin secretion was higher than in normal ones (Bodo *et al.*, 1997, Fig. 4B). Neither IL-1 nor IL-6 cytokine exerted any effect on fibronectin secretion by Crouzon or Apert cells.

Collagen synthesis

To investigate whether Crouzon and Apert periosteal fibroblasts selectively synthesise different types of collagen, ^{3}H labelled secreted α collagen chains were analyzed by SDS-PAGE using delayed reduction and quantified by densitometric scanning. The results showed that Crouzon fibroblasts secreted a greater amount of both type I and III collagens than normal cells (Bodo *et al.*, 1996, Fig. 5A). On the other hand, the amount of type I collagen secreted by Apert fibroblasts was about 60% that of normal cells, and the alpha 2/alpha1 ratio was reduced (Bodo *et al.*, 1997, Fig. 5B). Equivalent quantities of type III collagen were secreted by normal and Apert fibroblasts. The amount of the secreted collagens remained unchanged after IL-1 and IL-6 treatment of both Crouzon and Apert fibroblasts.

Glycosaminoglycans synthesis

Glycosaminoglycans were precipitated from supernatants obtained from Crouzon and Apert periosteal fibroblasts cultured in the presence of ^{3}H-glucosamine. Radioactivity was measured both in total precipitated GAG and in the fractions corresponding to specific GAG classes isolated by a DE-52 cellulose anion exchange column. As compared to normal fibroblasts, total newly

synthesised GAG was lower in Crouzon cells (Bodo *et al.*, 1996) and higher in Apert ones (Bodo *et al.*, 1997). Chromatography of aliquots of media revealed that hyaluronic acid (HA) was less expressed in Crouzon media, where a considerable increase in chondroitin sulphate (CS) was observed (Fig. 6A). The HA/total sulphated GAG ratio was shifted in favour of sulphated GAG. IL-1 and IL-6 significantly stimulated HA and heparin sulphate (HS) release, but depressed CS. The glycosaminoglycan profile in Apert fibroblasts revealed that the absolute levels of HA, HS, CS and DS rose without any change in the HA/sulphated GAG ratio; IL-1 and IL-6 differently modulated individual GAG classes, decreasing HA and increasing CS (Fig. 6B).

Response of Crouzon cells to FGF 2

To better understand the phenotypic consequences of interactions between FGF 2 and mutated FGF R 2 during membranous ossification, we analyzed the *in vitro* production of collagen and GAG in Crouzon osteoblasts treated with exogenous FGF 2 (Bodo *et al.*, 1999b). We also examined mRNA expression of PG biglycan, decorin, betaglycan and syndecan, and their regulation by FGF. The results showed that FGF 2 caused a drop of total GAG synthesis in Crouzon osteoblasts, and in particular, decreased cellular accumulation of HS and CS GAG chains (Bodo *et al.,* 1999b).

Crouzon osteoblasts showed only low traces of decorin transcript, which were unaffected by FGF 2; biglycan appeared significantly increased and downregulated by FGF 2 (Bodo *et al.*, 1999b, Fig. 7). The parallel observation that procollagen 1 gene is markedly expressed in Crouzon osteoblasts and significantly reduced by FGF 2 seems to indicate that FGF 2 specifically elicits a coordinated downregulation of collagen I, biglycan and decorin genes. Syndecan was more highly expressed in Crouzon than in normal osteoblasts, and its expression increased after FGF 2 treatment (Fig. 7). In contrast, no betaglycan was detected in Crouzon osteoblasts, irrespective of whether they were treated with FGF 2 or not. These findings may have a functional significance, since PG have a critical role in development and are involved in many genetic connective disorders (Ruoslahti, 1989, Pulkinnen *et al.*, 1990). Indeed, decorin and biglycan, which contain one and two chondroitin/dermatan sulphate GAG chains, respectively, interact with collagen types I, III and IV (Schonherr *et al.*, 1995, Svensson *et al.*, 1995). Betaglycan and syndecan are heparan sulphate/chondroitin sulphate PG that bind FGF 2 via their HS chains (Spivak-Kroizmen *et al.*, 1994; Szebenyi and Fallon, 1999). Several studies indicate that HS PG are required for FGF signalling, while others suggest that HS PG is only able to enhance the affinity of FGFs for FGF Rs (Roghani *et al.*, 1994). It has been hypothesised that HS PG may regulate specific FGF-FGF R interactions and determine which type of biological activity of FGF prevails (Aviezer *et al.*, 1997, Szebenyi and Fallon, 1999). It has been shown that PG production is cytokine-regulated (Tiedemann *et al.,* 1997), and in particular that FGF 2 modulates biglycan at the level of transcription, translation and sulphation (Kinsella *et al.*, 1997). For this reason, variations in cytokine expression influence the organization and properties of matrix by inducing changes in the relative composition of PG.

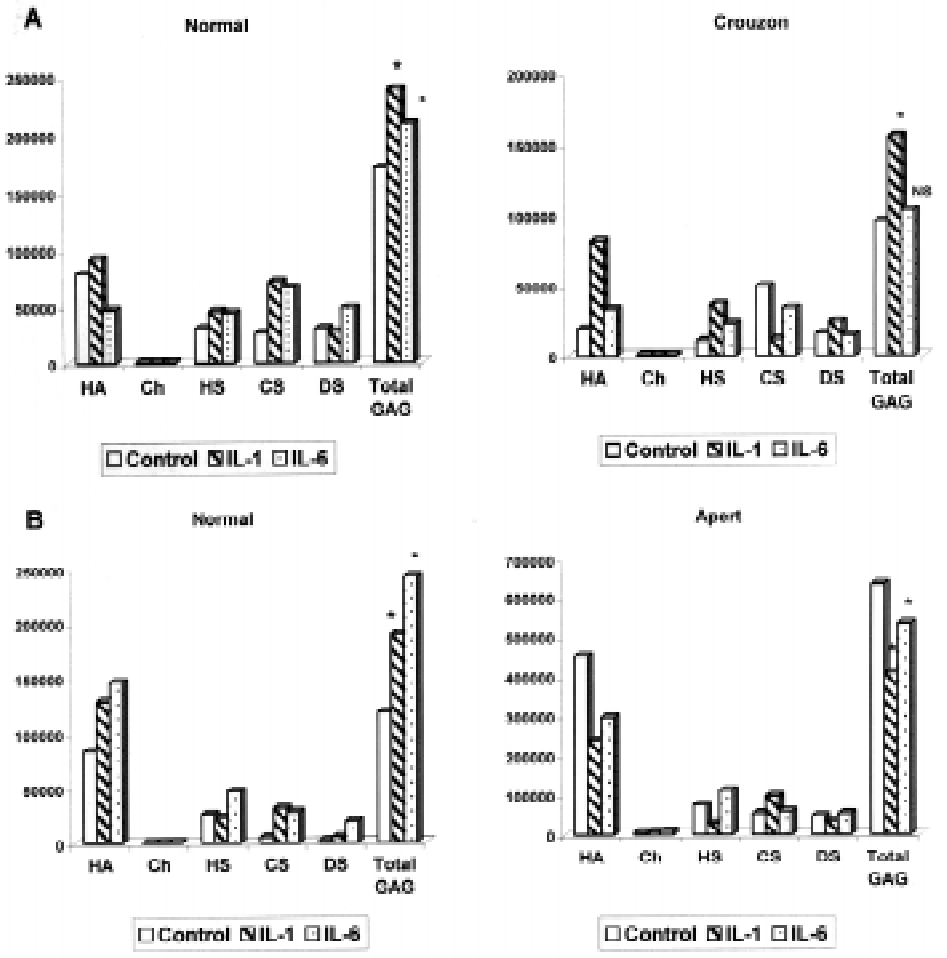

Fig. 6. [^{3}H]-glucosamine incorporation into total and individual GAG classes secreted by normal, Crouzon and Apert fibroblasts with or without IL-1 and IL-6 . *Normal and Crouzon fibroblasts were maintained for 48 h in MEM + 0.5% FCS with or without IL-1 or IL-6 (both 5 U/ml) and [3H]-glucosamine (s.a. 36.7 Ci/mmol) during the last 24 h. Aliquots of media were applied to DE-52 cellulose anion exchange columns to identify the individual GAG classes. Total GAG values represent the mean of four replicates from a typical experiment. Standard deviation was less than 15%. Differences from each control are significant for *P<0.001. NS = not significant. Chromatography values are representative of a single chromatography. Similar results were seen in three independent experiments. HA= hyaluronic acid; Ch = chondroitin; HS = heparin sulfate; CH = chondroitin-4 and -6 sulfate. DS = dermatan sulfate. (From Bodo et al., 1996, 1997).*

Conclusions

Taken together, these findings shows several ways in which the Apert and Crouzon phenotypes differ with respect to normal ones. Altered ECM production has been previously observed by Lomry *et al.*, (1998) in an immortalised cell line of Apert osteoblasts. These authors hypothesised that the FGF R 2 mutations present in

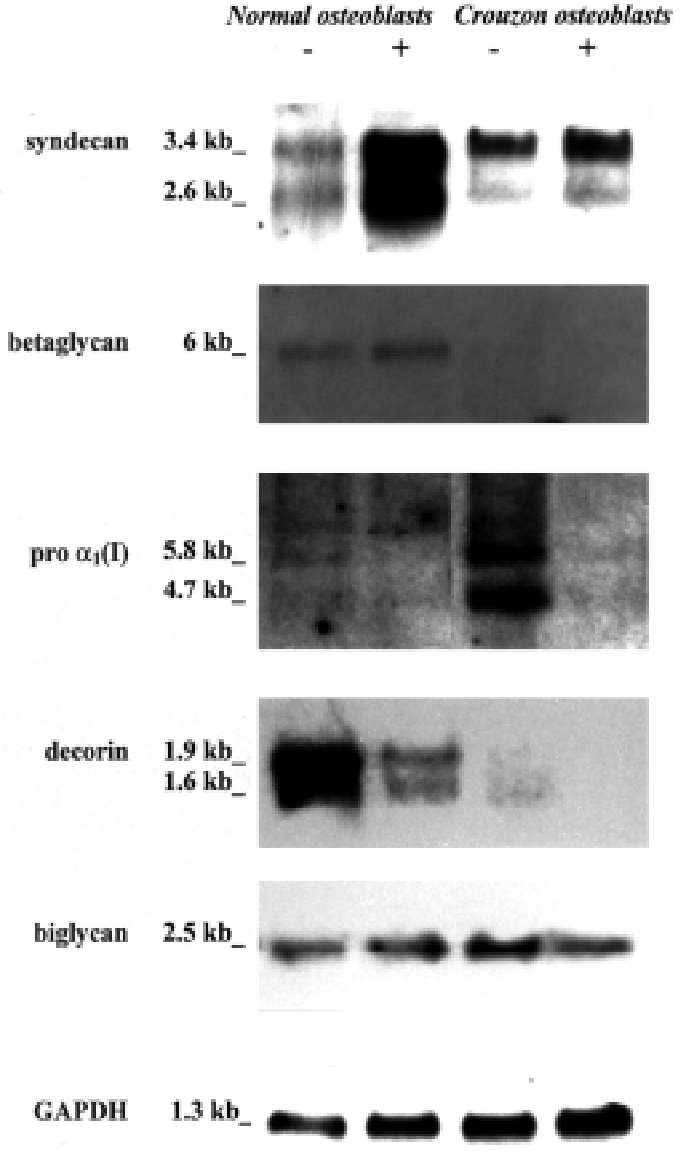

Fig. 7. Expression of syndecan, betaglycan, type I procollagen, decorin, biglycan and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in human normal and Crouzon osteoblasts in the presence (+) or absence (-) of FGF 2 (20 ng/ml). *Twenty micrograms of total RNA per lane were analysed using the Northern hybridisation technique. The same filter was stripped and re-hybridised with [^{32}P]-labelled cDNA probes as indicated.*

Apert patients increase the number of uncommitted precursor cells that enter the osteogenic pathways, leading to increased matrix formation and premature calvaria ossification. Nevertheless, the observation that FGF R 2 mutations appear to underlie many forms of craniosynostosis does not explain how mutations in identical or neighbouring positions result in clinically distinct syndromes like Apert and Crouzon. The greater clinical severity of Apert with respect to Crouzon syndrome may be related to the different *in vitro* phenotype observed by us. The cytokine-induced ECM modulation observed by us, suggests that in Crouzon and Apert cells other genes besides mutated FGF Rs may account for the phenotypic variability.

It also suggests the existence of several key genes involved in the regulation of skull development, pattern formation and morphogenesis. The sequence of events in craniofacial development is likely to be influenced by the relative balance of various cytokines at any given time rather than the absolute concentration of any particular one. Thus, Apert and Crouzon phenotypes may be associated with the persistence of positive feedback mechanisms of cytokine expression and autocrine/paracrine actions. Furthermore, the differences in cytokine expression between Apert and Crouzon cells may be related to different genetic backgrounds that influence the severity and penetrance of mutant phenotypes, and could explain the greater clinical gravity of the Apert syndrome. In addition to the hypothesis of this genetically-induced program, the changes observed in cytokine pattern and connective macromolecules production observed in Apert and Crouzon cells, could also be induced by a feedback system control directly caused from the alterations in ECM and cytokine production. The control of delivery and accessibility of cytokines and ECM components may therefore turn out to be another important mechanism in the control of the development processes. The observed increase in Crouzon osteoblasts of mRNA expression of biglycan and syndecan, respectively downregulated and upregulated by FGF 2, may affect the links between biglycan and TGFβ and between syndecan and FGF 2 respectively, thereby modifying the diffusion of the ligands in developing tissues. Further functional significance could be ascribed to the increase in syndecan expression in Crouzon osteoblasts, since syndecan is an HS-PG, a tissue-specific co-receptor for FGF 2. Taken together, these data suggest that the changes in the distribution of ECM components participate in the regulation of the complex morphogenetic events that occur during skull development. Several growth factors are involved in this cascade of events, each playing a role in the commitment of calvaria cells to different phenotypes. The balance among ECM components and cytokines probably determines the degree and extent of FGF 2-induced cellular response. Research into the mechanisms regulating this balance has entered an exciting phase, and cultures from Apert and Crouzon patients provide a promising model for these studies. One major challenge is to unravel the signal transduction pathway by which FGF 2 ligand receptor binding causes transcriptional activation of specific genes leading to premature calvaria ossification. Preliminary data obtained by our group seem to indicate alternative and different signalling pattern in Apert cells with respect to Crouzon ones (Volinia *et al.*, 1999). Further studies on cranial suture biology and pathology are necessary to show whether specific cytokine-directed therapeutic strategies can find a place in clinical practice as a complement to surgery.

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