Bull. Group. Int. Rech. Sci. Stomatol. Odontol. 45, 66-78, 2003

The Role of Fibroblast Growth Factor (Fgf) and Type β Transforming Growth Factor (Tgf-β1-β2-β3) during Rat Craniofacial Development.

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KEYWORDS: fibroblast growth factor, type β transforming growth factor, craniofacial development, skull sutures, rat development.
MOTS CLES: fibroblast growth factor, type β transforming growth factor, developpement craniofacial, sutures craniennes, developpement du rat.

ABSTRACT

Growth factors seem to be part of a complex cellular signalling language, in which individual growth factors are the equivalents of the letters that compose words. According to this analogy, informational content lies, not in an individual growth factor, but in the entire set of growth factors and others signals to which a cell is exposed. The ways in which growth factors exert their combinatorial effects are becoming clearer as the molecular mechanisms of growth factors actions are being investigated. A number of related extracellular signalling molecules that play widespread roles in regulating development in both invertebrates and vertebrates constitute the Fibroblast Growth Factor (FGF) and type β Transforming Growth Factor (TGF β). The latest research literature about the role and fate of these Growth factors and their influence in the craniofacial bone growth ad development is reviewed.

RESUME

Les Facteurs de Croissance (*Growth Factors)*, font aparémment partie d'un langage complèxe de signaux céllulaires, dans lequel les facteurs de croissance individuels séraint l'équivalent des lettres qui composeraient les différents mots. À la suite de cette analogie, le contenu de l'information ne se trouverait pas dans un facteur isolé, mais dans tout l'ensemble de facteurs de croissance et d'autres signaux auxquels la cellule est exposée. Les voies par lesquelles ces mollécules éxercent leurs effets combinés deviennent plus clairs au fûr et à mésure que les méchanismes d'action des facteurs de croissance sont décrits. Le Fibroblast Growth Factor (FGF) et le Transforming Growth Factor-β (TGFβ) constituent les deux groupes de mollécules de signalisation qui jouent un role plus étendu en la regulation du dévéloppement aussi des invertebrés que des vertebrés. Cette revue de la literature prèsente les derniers travaux sur le rôle et l'evolution de ces Facteurs de Croissance et leur influence sur la croissance et le dévéloppement des os du crâne et de la face.

INTRODUCTION

The biochemical variations in craniofacial sutures at differents stages of their development are still unknown. To understand the normal and abnormal behaviour such knowledge migth be crucial. A number of related extracellular signaling molecules that play widespread roles in regulating development in both invertebrates and vertebrates constitute the Fibroblast Growth Factor (FGF) and type β Transforming Growth Factor (TGFβ)[1-4]. TGF-β is a 25 kDa dimeric protein which represents a large family of factors with a variety of activities. The concept that TGF-β is prototypic of a superfamily of growth, differentiation, and morphogenetic factors became clear in 1987[3,4]. The structural prototype for this gene superfamily is the protein that was first isolated from human platelets as TGF-β [5], cloned from a human cDNA library[6] and later named TGF-β1[7]. The distribution of TGF-β related factors is widespread in organisms from fruit flies to humans, and their evolutionary conservation is unusually strict [8]. There are three TGF-β isoforms (TGF-β1-β3) that initiate a variety of osteogenic cascades in mammals[9-12].

The Fibroblast Growth Factor family currently comprises nine members, FGF1 to 9, which in mammals, can interact with four different receptor tyrosine kinases (FGF receptors [FGFRs]) to stimulate mitogenic, differentiation, migration and survival responses depending on the target cell and its development history[13-14]. The genes encoding FGFs and FGFRs are evolutionarily conserved and the receptors have been identified in both vertebrates and invertebrates[15]. Many FGF genes display expression patters in early mammalian embryos suggestive of significant role in development[16-22]. FGF promotes cell proliferation in chondrocytes in *vitro*[23] and in *vivo*[24] as well as in osteoblasts in *vitro*[25].

To understand the role of cranial sutures as intramembranous bone growth sites, its necessary to establish where sutures occurs, how they form, and what regulates their formation and maintenance[26] . Despite the obvious differences in human and rodent craniofacial characteristics, there is an amazing conservation in the molecular specification and assembly of the embrionic cranial structures. Coordinated allometric growth of the cranium is achieved through an elaborate series of tissues interactions among of the brain, dura mater, suture mesenchyme, and calvarial bones[27]. The presence or absence of FGFand TGFβ during embryonic mouse development may dictate wide-ranging effects on the progression of skull expansion.

If these studies elucidate the specific functions of FGFs and TGF-β members during mouse craniofacial development, and provide important molecular characterization, will constitutes a foundation for the design and testing of non-surgical therapies for the correction of abnormal craniofacial development in humans.

TRANSFORMING GROWTH FACTOR β AND BONE

Normal skeletal growth results from a balance between the processes of bone matrix synthesis and resorption. The biochemistry of skeletal growth is complex, and results from many anabolic and catabolic processes regulated by endocrine, paracrine, and autocrine factors. Although many growth promoters are associated with bone matrix, it is related particulary with TGF-β activity. The intricated mechanims by which TGF-β regulates bone formation are likely to be fundamental to understanding the processes of skeletal growth during development, maintenance of bone mass in adult life, and healing subsequent to bone fracture[28].

Both bone matrix and the conditioned medium of bone organ cultures are rich in TGF-β[29-34]. TGF-β is thought to be a local regulator of bone growth, as it has dose-dependent effects on cell replication in bone organ culture and in osteoblast-enriched cell populations from cultures of fetal rat calvaria[34-36]. In 1990 Shinar and Rodan reported a biphasic effect of TGF-β on the production of osteoclast-like cells (OC) in mouse bone marrow cultures; low concentrations of TGF-β stimulated the generation of OC, whereas higher concentrations inhibited greatly their production[38].

TGF β also regulates the expression of extracellular matrix proteins at the transcriptional and posttranscriptional levels in a variety of isolated cell cultures[8, 39-45]. In bone organ culture and osteoblast-enriched cell cultures, it alters differentiated cell funtion by stimulating protein and collagen synthesis and inhibiyig alkaline phosphatase activity and osteocalcin production [35,46-48].

Noda and Camilliere provided in 1989 the first evidence that all TGF-β stimulates bone formation in vivo[49]. Experimental evidence indicates that bone and cartilage contain large amounts of TGF-β and active

target cells for its activity [9]. In 1990, Joyce, *et al* [50] showed that the effect of TGF-β on the periosteum is first to stimulate the proliferation of mesenchymal cells that then differentiate into chondrocites and osteoblast. The net result of coninued TGFβ injection was the formation of a callus with central cartilage and lateral intramembranous bone; the endocondral replacement of the cartilage, and the remodeling into cortical bone after cessation of the TGF-β treatments. They showed that TGF-β2 induce a matrix that contains both type I and type II Collagen a characteristics that is compatible with Chondroid tissue, which was described by Goret-Nicaise[51]. Carrington *et al*[52], showed that TGF-β1 and β2 were present during endochondral bone development when the critical transition from calcified cartilage to bone was taking place in presence of osteoblasts. Hock *et al.*[53] showed that TGF-β is a mitogen in all cell zones of the pericranial periosteum. This mitogen effect is in contrast to the more specific effects of the Insuline Like Growth Factor (IGF-I) which preferentially stimulate cell replication in the progenitor and osteoblast cell zones [54].

TGF-β AND EMBRYONIC DEVELOPMENT

The history of the skull development is complicated because its progressive phylogenetical modifications and its adaptative specialization to the new and more complex functions that appear during the evolution[55]. The skeletal system develops from the paraxial and lateral plate (somatic layers) mesoderm and from the neural crest[56]. Cranial neural crest migration in the mouse begins at embryonic day 8 and is completed after approximately 2 days[27].

Neural crest cells in the head region differentiate into mesenchyme (*ectomesenchyme*) and participate in the formation of the face and skull bones. The more ancient and dorsally situated part of the skull base, that appears around the notochord, is from mesodermic origin, derived from the occipital somites. The rest of the cranial base, derived from the paraxial mesoderm, completes the cranial base structure, by way of an endochondral ossification process[55, 57]. The phylogenetically newest part of the skull develops from the neural crest cells. It constitutes both the exoskeleton of the neo-encephalon and the viscerocranium, thus including in a complete structure of mesectodermal origin both the calvaria, the bone structures surrounding the sensory organs, and the branchial arches derivatives, which will constitute the stomatognathic system. These neural crest cell derivatives develop into bones by way of an intramembranous ossification process[55].

In the rat, all cranial vault sutures with the exception of the posterior interfrontal suture remain patent for the life of the animal[27]. At birth the flat bones of the human skull are separated from each other by narrow seams of connective tissue, the sutures, which are also derived from neural crest[55]. Cranial vault sutures are the major sites of bone growth along the leading margins of the cranial bones during craniofacial development, especially during the rapid expansion of the neurocranium [58].

The posterior fontanelle closes about 3 months after birth, but the anterior fontanella normally remains open until 1.5 year of age[59]. The main biological funtion of the sutural tissue is not restricted to being an articulation but appears to include: 1) to unite bones, while allowing minor movements; 2) to act as growth areas; and 3) to absorb mechanical stress, thus protecting its osteogenic tissue[60] and the neural structures beneath.

Pritchard *et al*[61] stated that a difference exists in the development of facial and cranial sutures. Persson and Roy[62] offered a biomechanical explanation for the morphogenesis of facial sutures, where dura mater does not exist. Based on the sutural development of the rabitt palate, they concluded that spatial separation of bones during growth regulates suture formation. Whereas the bones in the facial skeleton approach each other in loose mesenchymal tissue, those in the cranial vault approach each other in an already differentiated membrane, the fibrous brain capsule. This capsule, the extomeninx, is delaminated by the osteogenesis into an outer periosteal layer and an inner dura mater.

To function as bone growth sites, sutures need to remain patent while allowing rapid bone formation at the edges of the bone fronts[27]. Cartilages adjacent to facial and cranial sutures occur both in humans[63] and in the midpalatal suture of rats and mice, where a synchondrotic union between bones is established[62,64]. The cartilages are secondary cartilages and are mainly found in rapidly growing areas. As for the calcified tissues, many authors[65-68] have attributed the presence of cartilage in the sutures to the relative anoxia and to the biomechanical forces present in the sutural area during its closure, even if they have also suggested that the normal sutural closure occurs without the participation of cartilaginous tissue. The presence of chondroid tissue in the human cranial vault, the biomechanical reasons for its presence and the precise role witch plays in the closure of the metopic suture in the human were already described by Goret-Nicaise and her co-workers[69-71] in the 1980´s. Rafferty and Herring[68] have recently confirmed

those findings in experimental studies.

TGF-β like activity has been reported in extracts of mouse embryos[72-73] by differential immunolocalization of all three TGF-β isoforms in embryonic cartilage (perichondrium and chondrocytes) and bone (periosteum and osteocytes), as well as in liver and thymus[74,75]. Findings from these studies indicated a complex interaction pattern of translation and secretion between the TGF-β isoforms existing in the mouse embryo. The results also suggested that the TGF-β isoforms act through paracrine and autocrine mechanisms during embryogenesis.

However, the intrinsic physiological role of those peptide in the developing embryo is still unknown. Heine *et al*[76] presented data suggesting that TGF-β has an important role in many embryonic tissues, particularly in those in mesodermal and neural crest origin, at specific times, when critical morphogenetic and histogenetic events occur.

They also showed that the participation of TGF-β in cartilage and bone formation is not limited to the axial skeleton, since intense staining is also found in craniofacial mesenchyme of neural crest origin, destined to become mandible, maxilla, palate, nose and nasal tissues, and other important craniofacial structures.

Mouse skulls have been studied histologically in order to better understand the morphogenesis and maduration of cranial sutures. Contemporany research has fundamentally reformed our understanding of cranial suture biology. In vitro and in vivo models demonstrated that the subjacent dura mater shapes spatially supplying osteoinductive growth factors (TGF-βs or FGF-2) and cellular elements (eg. Osteoblast-like cells) to the overlying osteogenic fronts and suture mesenchyme[77]. There is a differential biologic activity for TGFβ isoforms during cranial suture, development, growth and fusion.

ROLE OF TRANSFORMING GROWTH FACTOR IN CRANIAL SUTURE FUSION

Despite the high degree of homology among the three mammalin isoforms, TGF-β1, TGF-β2, TGF-β3 can have different biological effect on the same cell [78]. To determinate whether growth factors were involved in the development, growth and maintenance of sutures its necessary to determine their presence in suture tissues. Opperman *et al*[79] have demostrated the specific location of TGF-β1, β2 and β3 in developing sutures, sutures remaining unossified, and sutures becoming obliterated, thereby implicating a role for these growth factors in cranial suture morphogenesis. The continued presence of of TGF-β1 and β2 in the developing rat coronal and frontonasal suture proven by inmunohistochemical analysis, was associated with posterior frontonasal suture fusion, whereas a decrease in intensity in TGF-β1 and TGF-β2 inmunoreactivity was associated with the maintenance of coronal suture patency. Increased TGF-b3 activity was associated with the coronal suture remaining unossified, suggesting a role for TGF-β3 in maintaining suture patency. Roth *et al* [80,81] described the inmunolocalizatin of TGF-β1, TGF-β2 and TGF-β3 and IGF-I in both fusing and patent cranial sutures in rats and in human. In the rat, there was an increased inmunoreactivity for the TGF-β isoforms and for IGF-I in the actively fusing posterior frontal suture compared with patent control sutures. In an analysis of human suture specimens, a more intense immunoreactivity for these same growth factor was noted in the osteoblast at the margins of prematurely fusing sutures when compared with patent control sutures [79].

Numerous studies have shown that sutural fate (*ie.* Fusion *vs* Patency) is regulated by the dura mater directly underlaying the cranial suture[62,82-85] Menhara *et al*[83] using recombinant DNA technology, a replication deficient adenovirus encoding a defective TGF-β receptor capable of blocking TGF-β receptor to the dura mater underlying a cranial suture programmed to fuse can significantly attenuate suture fusion even after prolonged in vitro culture. Sagiroglu *et al*[86] demonstrated that TGF-β1 production is increased significantly in the postero-frontal suture compared with the sagittal suture in the initial stages of "in vitro" suture fusion, and that TGF-β1-3 production by these tissues decreased thereafter.

Studies supporting a role of dura mater in cranial suture fusion detail its osteoinductive and osteoconductive properties [87-89]. Postero-frontal suture surgically manipulated to overlie the sagittal suture dura mater remain patent, whereas sagittal sutures manipulated to overlie the postero-frontal suture dura mater fuse[50,90] In addition, postero-frontal sutures maintained separated in vitro from their underlying dura mater remain patent, whereas separation followed by coculture of postero frontal suture complex and its underlying dura mater restores programmed sutural fusion [91]. Yu *et al*[88] have shown that transplantation of calvarial dura mater into epitheliomesenchymal pockets can induce bone formation. Other investigations have shown that dura mater is the critical determinant of

calvaria bone regeneration after surgical excision in immature animals [92,93]. Spector *et al* [94], isolated 35 to 40 postero-frontal and Sagital suture complexes, and suture-derived cell cultures were established. They demostrated that molecular differences between the posterior frontal and sagittal suture complexes were readily identified *in vivo*, although these distinctions were lost once the cells comprising the suture complex were cultured *in vitro*. They suggested that these changes in gene expression resulted from the loss of the influence of the underlying dura mater. High levels of TGF-β1 inmunoreactivity were further detected in cells lining the bone fronts of coronal suture undergoing osseus obliteration after being cultured in the absence of dura mater: lower levels of inmunoreactivity were seen in nonfusing coronal sutures cutured in the presence of dura mater. In contrast to the in vivo findings, low levels of inmunoreactivity of TGF-β2 remained in coronal sutures cultured in both the presence and the absence of duramater.

Suture obliteration induced by removal of TGF-β3 activity was preceded by elevated levels of DNA synthesis, similar to these seen upon removal of the dura. Addition of exogenus TGF-β3 to calvarie cultured without dura both prevents suture obliteration and reduces DNA synthesis to levels comparable to those seen in with sutures intact dura. Addition of exogenous TGF-β2 to calvarial cultures induced sutural fusion accompanied by elevated levels of cell proliferation. Sutures rescue from obliteration by removal of TGF-β2 activity did not have decreased levels of cell proliferation [95].

The biological functions of TGF-βs are modulated by interactions with two widely expressed transmembrane serine-threonine kinase receptors: (Tb-RI and Tb-RII)[96]. Menhara *et al* [97] have show that Tb-RI and Tb-RII immunostainig is increased in the dura mater and osteoblast of the sutural margin of the postero-frontal suture during active suture fusion compared with the osteblast and dura mater underlying the patent sagittal suture. Cui *et al*[89] have also localized the expression of TGF-β3 and TB-RII in palatal epithelial cells during palathogenesis. Transforming growth factors-b3 does not regulate protein levels of TGF-β2 in sutures[94,90], so TGF-β3 could regulate tissue responsiveness to TFG-β2 by regulating TGF-β2 access to receptors.

Opperman *et al*[100], found that the numbers of cells expressing Tb-RI within the suture matrix increased over time in sutures remaining patent. Osteoblastic cells lining the bone fronts on either side of sutures were Tb-RI positive during early morphogenesis, but these numbers declined as sutures fused, both in *vivo* and in *vitro*. Addition of TGFb3 to calvaria in culture decreased the number of Tb-RI expressing cells in both fusing and non-fusing sutures, with dramatic decreases in the numbers of osteoblast expressing Tb-RI.

FIBROBLAST GROWTH FACTOR

Significant advances in our understanding of the later development roles of FGFRs have come from investigations into genetics basis of human dysmorphic diseases. These investigations have highlighted the critical role that the FGFr family plays in bone and limb development[101,102]. Mutations in FGFr3 have been shown to be responsible for achondroplasia, the most common form of dwarfism[103,104].

The achondroplasia phenotype is consistent with the FGFr3 RNA expression patters both in the developing mouse and human, which showed transcripts in the cartilaginous rudiments of all developing bones including the skull and long bones of the limbs[105,106].

Recent genetic mapping studies have demonstrated mutations in fibroblast growth factor receptors, particulary FGFr1 and FGFr2, in association with craniosynostotic syndromes[107,106]. Mutations in FGFr1 have been associated recently with Pfeiffer syndrome[113], an autosomal dominant disease characterized by craniosynostosis, or premature fusion of the cranial sutures, resulting in an abnormal skull shape and craniofacial anomalies.

Mutations in FGFr2 have also been identified in three other autosomal dominant craniosynostosis syndromes known as Jackson-Weiss, Crouzon and Apert syndromes[101]. In addition, numerous *in vitro* and *in vivo* studies have demonstrated an important role for basic fibroblast growth factor (bFGF), the most abundant FGFr ligand, in the regulation of ephitelial-mesenchymal interactions, limb pattering, fracture healing, and bone growth[114-116].

The analysis of targeted mutations in the genes encoding mouse FGFs and their receptors has demonstrated the critical role that these signalling molecules play in regulating embryonic growth and patterning during the early post-implantation development. The demonstration that mutations in human FGFrs underline several dysmorphic diseases has

provide genetic evidence that FGFrs are involved in limb and craniofacial development[101].

In developing cranial sutures both *in vivo* and *ex vivo*, the undifferentiated mesenchymal cells of the sutures have the dual role of proliferation within sutural tissues to maintain the normal suture cellularity and architecture while simultaneously providing a pool of cells from which osteoblast, along the osteogenic fronts, can differentiate to form bone [89]. Several candidate factors are present during the development and growth of cranial sutures and likely play a part in this complex regulatory sequence. Through the identification of mutations in their receptors, the fibroblast growth factors (FGFs) have similarly been implicated in suture regulation[89]. Opperman et al[79] have demonstrated that embryonic cranial suture patency in vitro is dependent on the presence of the underlying dura and that this interaction is mediated by soluble, heparin biding factor such as the fibroblast growth factor. Also they were the first to suggest the existence of communicators for the suture-dural interactions that maintain cranial suture patency during development.

The fact that bFGF is a heparine binding molecule, together with the finding that increased bFGF immunostaining in the fusing posterior frontal suture was initially noted in cells of the underlying dura mater and osteoblast of the endocranial bone. This was the observation of followed later by increased staining of the remaining sutural connective tissue, that suggests that bFGF is an important regulator of bone induction and sutural fusion by the dura mater[117]. Spector *et al*[94] reported that stimulation of immature dura mater with recombinant human FGF2 resulted in significant changes in proliferation, expression of extracellular matrix molecules and markers of the osteoblast lineage, and production of osteoinductive cytokines. This incresead pool of more differentiated cells may be a crucial effector of calvarial morphogenesis, cranial suture fusion and reossification after injury. FGF2 levels are highly elevated in fusing sutures[118] moreover addition of FGF2 to sutures induces fusion[119]. Also in addition of FGF4 to cultured foetal mouse calvaria induces premature suture fusion associated with elevated levels of cell proliferation[120].

Normal human osteoblast increase TGF-β2 production during prolonged exposure to FGF2, accompanied by increased osteoclast production and matrix mineralization[121]. Opperman[43] suggests that most of the FGFr mutations do not affect proliferative activity, but rather alter cell differentiation. Increased cell proliferation at the suture, both increasing the bone cell lineage and accelerating osteoblast differentiation, results in increased bone formation, that would be sufficient to induce premature suture obliteration. Experimental evidence supporting receptor autoregulatory mechanism in fibroblast growth factor-mediated mechanisms can be derived from studies demonstrating regulation of FGFr1 and FGFr2 in response to fibroblast growth factor stimulation[122-125].

Meharara[117] *et al* and also Most *et al*[118], have demonstrated increased bFGF and decreased FGFr1 and FGFr2 immunostaining during sutural fusion in the rat posterior frontal suture. They have demonstrated an increased FGFr1 and FGFr2 immunostaining in the patent sagittal suture as compared with the fusing posterior frontal suture. A similar pattern of immunostaining was noted in experiments investigating FGFr2 in patent and synostotic sutures of patients with Crouon syndrome[125,126].

Those data suggest an active role for bFGF in the regulation of sutural closure. Recently, Iseki *et al.* [119] have investigated the patters of expression of FGFr1, FGFr2 and FGFr3 in the fetal mouse head, with specific reference to their relationship to cell proliferation and differentiation in the frontal and parietal bones and in the coronal suture. FGFr2 is expressed only in proliferating osteoprogenitor cells; the onset of differentiation is preceded by down regulation of FGFr2 and up-regulation of FGFr1. Following up-regulation of the differentiation marker osteopontin, FGFr1, osteonectin and alkaline phosphatase are down-regulated, suggesting that they are involved in the osteogenic differentiation process but not in maintaining the differentiated stated. They suggest that a gradient of FGF ligand, from high levels in the differentiated region to low levels in the enviroment of the osteogenic stem cells, modulates differential expression of FGFr1 and FGFr2 and that signalling through FGFr2 regulates stem cell proliferation whereas signalling through FGFr1 regulates osteogenic differentiation.

Warren *et al*[127] showed that *noggin*, an antagonic of bone morphogenetic proteins (BMPs) required for embryonic neural tube, somites and skeleton patterning[128-130] is expressed postnatally in the suture mesenchyme of patent but not fusing cranial sutures, and that *noggin* expression is supressed by FGF2 and syndromic FGF-r signalling. Since *noggin* misexpression prevents cranial suture fusion *in vitro* and *in vivo*, they suggested that syndromic FGFr mediated craniosynostosis may be the result of inappropiated downregulation of *noggin* expression.

The analysis of targeted mutations in the genes encoding mouse FGFs, TGFs and their receptors has demostrated the critical role that these signalling molecules play in regulating embryonic growth and pattering during the early post-implantation development.

The mouse embryo presents a hard challenge to development biologists. It has however two immediate attractions. First, the mouse is a mammal as humans are. Second, among mammals, it is one of the most convenient for genetics studies, because it is small and breeds rapidly.

These two factors have spurred an enormous research effort, resulting in the development of some remarkably powerful experimental tools that will hopefully help us to understand the complicate process of cranial growth in the future .

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