Advances In Murine Cranial Suture Research

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Abstract

Craniosynostosis is a pathologic condition that results from premature fusion of one or more cranial sutures. It occurs in approximately 1:2000 live births. Since the brain expands rapidly in the first few years of life, premature closure of a cranial suture leads to compensatory calvarial expansion in a plane parallel to the fused suture. Untreated, craniosynostosis can cause a characteristic dysmorphic calvarial shape, midface hypoplasia, and can lead to deafness, blindness and mental retardation. In order to understand the dynamic mechanisms that mediate craniosynostosis, we needed to investigate the biologic processes, before, during and after suture fusion. Since clinical specimens limit our investigation to the time at which the samples are excised, we have employed murine models to examine the cascading events that lead to cranial suture fusion. These models have enabled us to dissect, isolate and understand the individual roles of the dura mater, pericranium, suture mesenchyme and osteogenic fronts.

INTRODUCTION

Craniosynostosis is a pathologic condition that results from premature fusion of one or ore cranial sutures. It occurs in approximately 1:2000 live births. [1] Since the brain expands rapidly in the first few years of life, premature closure of a cranial suture leads to compensatory calvarial expansion in a plane parallel to the fused suture. Untreated, craniosynostosis can cause a characteristic dysmorphic calvarial shape, midface hypoplasia, and can lead to deafness, blindness and mental retardation. [2]

Although unilateral non-syndromic coronal synostosis is most common, more than 150 genetic syndromes have been described. [3] Premature suture fusion may also be caused by hyperthyroidism, hypophosphatemic vitamin D-resistant rickets, mucopolysaccharidoses and mucolipidoses. [4,5,6,7,8] In spite of its association with a number of syndromes and metabolic disorders, the etiopathogenesis of craniosynostosis remains unknown.

Although murine and human craniofacial characteristics are obviously different, there appears to be tremendous conservation in the assembly of embryonic cranial structures.[9] We are exploiting this conservation to examine the molecular mechanisms that mediate programmed murine cranial suture fusion. Although it remains to be proven, we would speculate that mouse and man share similar calvarial molecular specification and sutural biology. The following series of experiments performed in our laboratory illustrate some of the advances in murine cranial suture research and highlight our understanding of the molecular mechanisms governing this system.

THE DURA MATER GUIDES CRANIAL SUTURE FATE

In order to understand the dynamic mechanisms that mediate craniosynostosis, we needed to investigate the biologic processes, before, during and after suture fusion. Since clinical specimens limit our investigation to the time at which the samples are excised, we have employed murine models to examine the cascading events that lead to cranial suture fusion. These models have enabled us to dissect, isolate and understand the individual roles of the dura mater, pericranium, suture mesenchyme and osteogenic fronts.

THE MURINE MODEL OF CRANIAL SUTURE FUSION

First, we defined the temporal sequence of cranial suture fusion in our models. By serially sectioning murine calvaria, our laboratory and others have demonstrated that the posterior frontal (PF) suture fuses in an anterior to posterior and endocranial to ectocranial direction from postnatal days 12-22 in the rat and 25-45 in the mouse.[10,11,12] We feel that this PF suture is analogous to the human metopic suture. In addition, we have demonstrated that all other cranial sutures, including the coronal (COR) and sagittal (SAG), remain

patent for the life of the animal. The disparate fate of these sutures was opportune because it enabled us to compare and contrast gene and protein expression in fusing and patent sutures.

Second, we developed an in vitro organ culture model to study cranial development in an isolated, serum-free system.[13, 14] This system was unique because it allowed us to eliminate independent variables from the cranial suture microenvironment. For example, extirpation of the cranial suture complex permitted normal in vitro calvarial development without extra-sutural variables (e.g. tensional forces or endocrine hormones) and provided a well-controlled environment for genetic modification and therapeutic intervention.[15]

Finally, using loupe magnification and micro-dissection, we established enriched cranial suture-associated dural and neonatal calvarial osteoblast cell lines.[16,17,18] These cell lines enabled us to understand the dura mater-derived signals and their effects on osteoblast phenotype.

The role of the dura mater in cranial suture fusion

In 1996, Roth et al. investigated dura mater-suture communication by studying the effects of PF cranial suture separation from the subjacent dura mater with an intervening impermeable silicone membrane.[19] In this experiment, Sprague-Dawley rats were divided into four groups. The control animals had no operation. Experimental animals underwent craniotomy alone, PF dural elevation only or silicone membrane interposition between the PF suture and the underlying dura. As expected, unoperated animals and animals that underwent craniotomy alone, demonstrated normal PF suture fusion. Animals that underwent PF dural reflection alone initiated delayed suture fusion on postnatal day 22 and completed ossification by postnatal day 30. Finally, the PF suture of experimental animals with dura mater-suture silicone separation remained patent through the period of predicted suture fusion (i.e. postnatal days 12-22). These animals did not initiate PF suture fusion until postnatal day 30.

We were surprised by these data because they suggested that the dura mater played an essential role in guiding cranial suture fate. Furthermore, we hypothesized that the PF dura mater was secreting soluble factors that were prevented from diffusing into the overlying cranial suture by the impermeable silicone membrane. This lead us to explore programmed regional specialization of the PF vs. SAG dura mater.

Regional specialization of the dura mater

In order to investigate the regional specialization of the dura mater, Levine et al. rotated the PF and SAG sutures with respect to the underlying dura. Sprague-Dawley rats were divided into two groups.[20] The control group underwent rectangular craniotomy from the lambdoidal suture to the jugum limitans inclusive of the PF and SAG sutures. The calvarium was separated from the underlying dura mater and then placed back on the dura mater in its original orientation. The experimental animals underwent the same procedure, except the excised strip craniotomy was rotated 180 degrees around the mid-sagittal axis. This rotation placed the PF suture over the SAG dura mater and the SAG suture over the PF dura mater. The control animals demonstrated normal suture physiology: the SAG suture remained patent while the PF suture completed normal anterior-posterior and endocranial-ectocranial ossification. In marked contrast, the PF suture (overlying sagittal dura mater) of experimental animals remained patent while the SAG suture (overlying posterior frontal dura mater) fused. Furthermore, the rotated SAG suture appeared to follow an anatomic anterior-posterior (overlying SAG suture posterior-anterior) and endocranial-ectocranial pattern of ossification.

Bradley et al. corroborated Levine's results with an in vitro mouse cranial suture organ culture system.13, 14 Bradley's in vitro rotational and translocational cranial suture data reemphasized the regional specialization of the underlying PF and SAG dura mater, but, moreover, the data implied that dura mater-suture communication, at least in postnatal development, was not dependent on tensional forces or distant endocrine hormones.

Characterizing the regional specialization of the PF and SAG-derived dural cells

In order to explore the regional difference in dural cells, we isolated the PF and SAG sutures of Sprague-Dawley rats.16 The underlying suture-associated dura mater was dissected free of the overlying suture complex and individual PF and SAG dural cell lines were established. First-passage SAG suture-derived dural cells demonstrated decreased cellular contact inhibition and significantly increased rates of cellular proliferation when compared to PF dural cells. In contrast, PF dural cells expressed more than twice as much alkaline phosphatase activity and collagen I protein. The PF and SAG dural cells both possessed the capacity to form bone nodules.

Collectively, these data demonstrated that phenotypic differences exist between early-passage dural cells derived from fusing and patent sutures. The formation of bone nodules suggests that both PF and SAG dura mater contain a population of osteoblast-like cells; however, elevated collagen I protein expression and alkaline phosphatase activity in PF dural cells suggest that the PF dura may contain more mature osteoblast-like cells. Cellular maturation and differentiation of PF dural osteoblast-like cells may be responsible for decreased cellular proliferation and enhanced contact inhibition.

The differences identified in suture-specific dural cells, in conjunction with the rotational and translocational cranial suture data, supported the hypothesis that the murine dura mater was regionally differentiated and provided paracrine signals to the overlying murine suture complex. Furthermore, the increase alkaline phosphatase activity and bone nodule formation in the PF dura suggested that this tissue contained a sub-population of osteoblastic cells that was markedly attenuated in SAG dura. Although it remains to be proven, we hypothesized that this sub-population of osteoblastic cells was contributing to PF suture fusion.

THE PERICRANIUM AND CRANIAL SUTURE MESENCHYME DO NOT CONTROL CRANIAL SUTURE FATE

Moss was the first to investigate the role of the pericranium.[21] Stripping the pericranium from neonatal rat calvaria, he observed normal PF suture fusion and COR suture patency. Opperman et al. added to Moss's findings by demonstrating that removal of the pericranium did not affect fetal or neonatal suture fate.[22]

By analyzing the gene expression within the pre-fusing, isolated cranial suture complex, Spector et al. have demonstrated that, like the pericranium, the intercalary suture mesenchyme appears not to participate in osteoinductive signaling; instead, it remains primed awaiting molecular instructions from the underlying dura mater.[23] In order to demonstrate this, six-day-old Sprague-Dawley rat calvaria were harvested and the subjacent dura mater and overlying pericranium removed. The isolated PF and SAG sutures were separated and either snap frozen and homogenized or digested with collagenase and used to establish early-passage suture-derived cell lines. Extracellular matrix protein and growth factor mRNA expression was compared in the snap frozen (in vivo) PF and SAG sutures. Identical analysis was performed in the established in vitro PF and SAG suture-derived cells. Snap frozen PF sutures expressed significantly more collagen I□I, collagen III and osteocalcin transcript than SAG sutures. In contrast, the level of TGF-?? mRNA was equal between the snap frozen PF and SAG suture complexes. These initial results implied that the pre-fusing PF suture complex does not intrinsically express critically important osteoinductive cytokines. Instead, the PF suture mesenchyme appeared to upregulate osteoid and ECM gene expression in response to inductive dura-derived signals.

Taken together, these experiments suggested that the osteogenic machinery within the isolated cranial suture complex remained primed awaiting osteoinductive paracrine signals from the underlying dura mater. These results lead us to investigate the nature of the dura mater-derived paracrine signals in the following series of experiments.

GROWTH FACTOR EXPRESSION IN CRANIAL SUTURE BIOLOGY

While the dura mater, independent of cranial base forces, appeared critical in determining sutural fate, the precise mechanisms mediating the dura mater-suture interaction remained unknown. In order to investigate dura-suture cytokine communication, we used a candidate gene approach. By in situ hybridization and immunolocalization techniques, we identified a number of osteogenic factors in the dura mater underlying the fusing PF suture. Furthermore, we identified very low-level expression of these same cytokines in the patent SAG suture. Finally, based on our understanding of gene expression in fusing vs. patent sutures, we were able to change the fate of programmed sutures by modulating the expression of these candidate cytokines.

The expression of insulin-like growth factors

The insulin-like growth factors (IGF-I and IGF-II) are 7.6 and 7.5 kD dimeric peptides, respectively.[24, 25] Both IGF-I and IGF-II are involved in bone formation and repair.[26,27,28,29] For example, The IGFs exert their mitogenic effects and induce collagen synthesis in osteoblasts through IGF type I and II receptors. Numerous studies have demonstrated that IGF I and IGF-II enhance bone healing when injected locally or even administered systemically.[30,31,32,33,34,35] Interestingly, Canalis and Lian demonstrated that IGF-I and IGF-II stimulate osteoblasts to express osteocalcin.[36] Since osteocalcin is expressed only by mature osteoblasts, the authors hypothesized that IGFs drive osteoblast differentiation.

In order to determine if IGF-I and IGF-II played a role in PF suture fusion, Bradley et al. harvested calvaria from Sprague-Dawley rats (ages: gestational day 16 to postnatal day 80), and examined IGF-I, IGF-II and osteocalcin expression.[37] The authors demonstrated that IGF-I and IGF-II mRNA and protein were exclusively expressed in the fusing PF dura mater and suture mesenchyme. The transcript and protein appear just before the onset of PF suture fusion (postnatal day 2-10) and persisted until the suture had completed fusion (postnatal day 30). Interestingly, the authors discovered that osteoblasts in the PF suture complex expressed marked amounts of osteocalcin. The authors hypothesized that dura-derived IGF-I and IGF-II were acting on the overlying osteoblasts to increase their rate of differentiation and osteocalcin expression.

The expression of transforming growth factor-betas and their receptors

The transforming growth factor beta (TGF-() superfamily includes a number of important growth factors including three TGF-(isoforms, the bone morphogenetic proteins, activins, inhibins, and growth and differentiation factors. TGF-(1, -(2, and -(3 are three closely related isoforms that are widely expressed during skeletal morphogenesis and bone repair.[38,39,40,41] These TGF-?s stimulate osteoblast proliferation and induce the synthesis of collagen, osteocalcin and other extracellular matrix proteins.[42,43,44,45,46] In addition, TGF-?s enhance extracellular matrix depostition by inhibiting osteoclast activity and down-regulating the expression of tissue metalloproteinases.[47,48,49,50,51] Furthermore, exogenous TGF-?1 enhances bone deposition and the healing of bone defects.[52,51,52,53,54,55]

Using in situ hybridization, a number of authors have localized TGF-?1 mRNA production to the dura mater underlying the PF suture.[56, 57] Collectively, these studies demonstrated that TGF-?1 transcription increased just prior to PF suture fusion. In marked contrast, the dura mater underlying the patent SAG suture expressed little TGF-?? transcript throughout the period of predicted suture fusion (i.e. 12-22 days). Interestingly, immunohistochemistry demonstrated that the elevated TGF-?1 transcripts in the PF dura are translated into protein that, we hypothesize, diffuses into the suture mesenchyme of the pre-fusing and fusing PF suture.12, 57, [58] Additional work has immunolocalized TGF-? receptors I and II (TGF-?R I and II) to osteoblasts in the PF osteogenic front and in the dura mater subjacent to the actively fusing PF suture. We hypothesize that TGF-?s are acting in a paracrine fashion to drive osteoblast differentiation and suture fusion.[59]

In 1997, Roth et al. demonstrated a differential TGF-? isotype expression pattern in human, nonsyndromic, unicoronal craniosynostotic sutures using immunohistochemistry.[60] The authors demonstrated a marked increase in TGF-?1 and ?2 growth factors in the osteogenic front of prematurely fusing sutures. In contrast, the patent sutures expressed minimal TGF-?1 or ?2. Interestingly, TGF-?3 protein production was limited to the sutural margin of the patent sutures. This restricted expression may implicate TGF-?3 in the suppression of osteogenesis.[61]

Taken together, the human craniosynostotic findings and murine data implicate TGF-? signaling in the regulation of cranial suture fate. In addition, the similarities in TGF-? isotype expression support the supposition that programmed suture fusion in murine models and premature fusion in man share, at least in part, evolutionarily conserved signaling pathways. Additional work is necessary to clarify the complex roles of TGF-?s in cranial suture biology.

The expression of fibroblast growth factors and their receptors

The FGFs are a large family of at least 19 cytokines that regulate cell migration, angiogenesis, bone development and repair, and epithelial-mesenchymal interactions.[62,63,64,65,66] FGF-2 is the most abundant ligand and it has been shown to stimulate osteoblast proliferation and enhance bone formation in vivo and in vitro.[67,68,69] FGF-2 expression is elevated in fracture healing and exogenously applied FGF-2 accelerates osteogenesis in critical size bone defects and fracture sites.[70,71,72] Furthermore, the FGF-2 signaling cascade augments the expression of TGF-? and its myriad of pro-osteogenic effects.[73]

Most and Mehrara et al. have spatially and temporally localized the expression of FGF-2 mRNA and protein during rat calvarial morphogenesis and PF suture fusion.56, 57 In situ hybridization revealed an abundance of FGF-2 transcript in the PF dura mater prior to and during PF suture fusion. In contrast, there was a paucity of FGF-2 mRNA in the SAG dura throughout the period of predicted suture fusion.56 Furthermore, immunohistochemistry demonstrated marked increases in FGF-2 protein production in the osteogenic front of the pre-fusing and fusing PF suture.[74] The spatial and temporal expression of FGF-2, in addition to the

experimental evidence supporting its regulatory roles in osteogenesis and epithelial-mesenchymal interactions, strongly implicates FGF-2 in the regulation of calvarial bone induction.

Since gain-of-function FGF receptor (FGFR) mutations are the most common syndromic cause of craniosynostosis, our laboratory and others have investigated the expression of FGF receptors in the murine model. Mehrara et al. demonstrated increased FGFR1 and FGFR2 immunostaining in the patent SAG suture compared to the PF. The authors hypothesized that the high FGF-2 ligand environment in the PF suture was down regulating the expression of FGFRs in that suture. Iseki et al. went on to investigate the different roles of FGFR1, FGFR2, and FGFR3 during mouse calvarial development.[75, 76] The authors have shown that FGFR2 expression coincided with areas of rapid cellular proliferation, but was mutually exclusive with domains of osteoblast differentiation. In contrast, FGFR1 expression was associated with osteoblast differentiation. FGFR3 was expressed in both the osteogenic and chondrogenic regions of the skeleton, including the thin plate of cartilage underlying part of the coronal suture, suggesting a cooperative role between FGFR2 and FGFR3 signaling in osteogenic cell proliferation. Additional work suggested that excessive FGF-2 signaling resulted in osteogenic differentiation and reciprocal FGFR2 down-regulation.75 This finding was important because it implied that differential FGF signal intensity may have qualitatively distinct cellular consequences.

Taken together, these studies suggested that FGF signaling played an important role in cranial suture biology.

Changing cranial suture fate.

Since accumulating evidence suggested that FGFs critically regulated cranial suture physiology, we attempted to determine if we could reverse programmed cranial suture fate by manipulating FGF-biologic activity. In order to do this, Greenwald et al. utilized replication-deficient adenoviruses encoding a truncated form of FGF-R1 (AdCAFGF-TR) or a secreted form of FGF-2 (AdCAsFGF-2).15 The AdCAFGF-TR construct was designed to abrogate FGF-biologic activity, while the AdCAsFGF-2 resulted in a marked increase in FGF-2 protein production. These constructs were injected into the PF (AdCAFGF-TR) or COR (AdCAsFGF-2) dura mater of embryonic day 18 Sprague-Dawley rats and the animals were examined on postnatal day 30. The authors demonstrated that in utero AdCAFGF-TR infection of the dural tissues underlying the PF cranial suture inhibited programmed cranial suture fusion, while in utero AdCAsFGF-2 infection of the dural tissues underlying the COR suture resulted in fusion of this normally patent suture. Through a variety of in vitro analyses, Greenwald et al. demonstrated that these effects were mediated via alterations in cellular proliferation, extracellular matrix molecule gene expression, and TGF-?1 synthesis. These data provided direct support for the hypothesis that FGF-biologic activity is a critical regulator of both programmed and pathologic cranial suture fusion

CONCLUSIONS

Normal cranial suture biology in murine models is very complex and seems to require a coordinated cascade of molecular signals from the underlying dura mater. While our knowledge of these dura-derived signals has increased dramatically in the last decade, we have barely begun to understand the fundamental mechanisms that mediate cranial suture fusion or patency. Ultimately, by understanding the mechanisms that mediate murine cranial suture biology, we may someday intelligently develop targeted biologically based strategies to treat or reverse prematurely fusing sutures.

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