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Indian and sonic hedgehogs regulate synchondrosis growth plate and cranial base development and function

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Abstract

The synchondroses consist of mirror-image growth plates and are critical for cranial base elongation, but relatively little is known about their formation and regulation. Here we show that synchondrosis development is abnormal in *Indian hedgehog*-null mice. The $Ihh^{-/-}$ cranial bases displayed reduced growth and chondrocyte proliferation, but chondrocyte hypertrophy was widespread. Rather than forming a typical narrow zone, $Ihh^{-/-}$ hypertrophic chondrocytes occupied an elongated central portion of each growth plate and were flanked by immature collagen II-expressing chondrocytes facing perichondrial tissues. Endochondral ossification was delayed in much of the $Ihh^{-/-}$ cranial bases but, surprisingly, was unaffected most posteriorly. Searching for an explanation, we found that notochord remnants near incipient spheno-occipital synchondroses at E13.5 expressed *Sonic hedgehog* and local chondrocytes expressed *Patched*, suggesting that Shh had sustained chondrocyte maturation and occipital ossification. Equally unexpected, $Ihh^{-/-}$ growth plates stained poorly with Alcian blue and contained low aggrecan transcript levels. A comparable difference was seen in cultured wild-type versus $Ihh^{-/-}$ synchondrosis chondrocytes. Treatment with exogenous Ihh did not fully restore normal proteoglycan levels in mutant cultures, but a combination of Ihh and BMP-2 did. In summary, Ihh is required for multiple processes during synchondrosis and cranial base development, including growth plate zone organization, chondrocyte orientation, and proteoglycan production. The cranial base appears to be a skeletal structure in which growth and ossification patterns along its antero-posterior axis are orchestrated by both Ihh and Shh.

Keywords: Indian hedgehog; Sonic hedgehog; Cranial base; Synchondrosis; Growth plate; Skeletogenesis; Extracellular matrix; Aggrecan; BMPs

Introduction

The cranial base is a morphologically complex portion of the skull that functions as a supporting platform for the developing brain, provides special physical niches to organs such as the pituitary, and establishes distinct articulations with different skeletal elements, including the mandible. The central region of the cranial base derives from the prechordal, hypophyseal, and parachordal cartilaginous plates, three pairs of precursor structures that form between the first and second month of gestation in humans (Larsen, 2001; Sperber, 2001; Thorogood, 1988). The plates are arranged in series, lie below the developing brain, and eventually fuse to form an uninterrupted cartilaginous structure spanning the region from foramen magnum to interorbital junction. With further development, primary ossification centers emerge within the unified cartilaginous structure, first posteriorly and then anteriorly (Kjaer, 1990). The major cartilaginous segments persisting between the ossification centers represent the synchondroses, termed ethmoidal, intrasphenoidal, spheno-occipital, and intraoccipital according to their anatomical location. The cranial base synchondroses display a remarkable palindromic organization, each consisting of two mirror-image growth plates arranged in opposing directions. The

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two growth plates share a central reserve/resting zone, display zones of chondrocyte proliferation, pre-hypertrophy and hypertrophy and endochondral ossification, but lack an articular synovial layer that is instead typical of growth plates in developing long bones. Given this orientation, the synchondroses produce growth in opposing directions and are responsible for lengthening and ossification of the cranial base mainly along its anterior–posterior axis (Mooney et al., 1993). Recent studies have provided some insights into the cell and molecular basis of synchondrosis development and growth (Rice et al., 2003; Shum et al., 2003; Gakunka et al., 2000; Chen et al., 1999; Ishii-Suzuki et al., 1999), but much remains unclear, particularly at the molecular signaling level.

Indian hedgehog (Ihh) is a critical regulator of skeletogenesis. Studies on long bone development have shown that *Ihh* is expressed in the pre-hypertrophic zone of growth plates (Vortkamp et al., 1996; Koyama et al., 1996a; Bitgood and McMahon, 1995). We found that *Ihh* expression coincides with ossification around the diaphysis and that exogenous Ihh promotes osteogenic cell differentiation in vitro, leading us to propose that a key role of Ihh is to initiate ossification in perichondrial tissues (Nakamura et al., 1997; Koyama et al., 1996a). Others found that Ihh limits the rates of growth plate chondrocyte maturation in concert with periarticular derived parathyroid hormone-related protein (PTHrP) (Lanske et al., 1996; Vortkamp et al., 1996). Studies with $Ihh^{-/-}$ mice provided clear support for both roles and indicated also that Ihh has a role in chondrocyte proliferation (Chung et al., 2001; St-Jacques et al., 1999). Currently, the view is that Ihh is a key paracrine regulator of distinct growth plate events in developing long bones: it stimulates chondrocyte proliferation and inhibits maturation together with periarticular tissue-derived PTHrP and induces intramembranous and endochondral ossification (Kronenberg, 2003). The present study was conducted to determine whether Ihh regulates growth plate function in cranial base synchondroses as well.

Materials and methods

Mouse embryo genotyping and anatomical analysis

Heterozygous $Ihh^{+/-}$ mice in F1 mixed background (129/SV:C57Bl6/J) were mated to generate homozygous $Ihh^{-/-}$ embryos. Fragments of embryonic and neonatal tails were genotyped by PCR using the following primers: for wild-type *Ihh* exon 1, forward primer 5′ CACCCCCAACTACAATCCCGACATCA 3′ and reverse primer 5′ AATGACCAGGGCTGGGCTGTGAGAA 3′ generating a 464 bp product; and for neomycin gene, forward primer 5′ AGGAGGCAGGGACATGGATAGGGTG 3′ and reverse primer 5′ TACCGGTGGATGTGGAATGTGTGCG 3′ generating a 300 bp product. Thermocycler conditions were 95°C for 30 s, 64°C for 1 min and 72°C for 1 min for 38 cycles. A total of over 400 wild-type and mutant embryos and newborns were analyzed in the study. Skeletons were stained with Alcian blue (Inouye, 1976), cranial bases were photographed and measured microscopically. Statistical analyses were performed by one way ANOVA with Tukey post hoc comparisons.

Gene expression analyses

For in situ hybridization, paraffin-embedded serial tissue sections were pretreated with 1 μg/ml proteinase K (Sigma) for 1 min at 37°C, post-fixed in 4% paraformaldehyde, washed with PBS containing 2 mg/ml glycine, and treated with 0.25% acetic anhydride in triethanolamine buffer (Koyama et al., 1996a). Sections were hybridized with antisense or sense ^{35}S-labeled probes (approximately 1×10^6 DPM/section) at 50°C for 16 h. Mouse cDNA clones were: aggrecan (nucleotides 880–1733; NM_007424); histone H4C (nt. 549–799; AY158963); PTHrP (nt. 66–1386; NM_008970); PTHrP-R (nt. 671–1450; NM_011199); osteopontin (nt. 1–267; AF515708); *Osterix* (nt. 40–1727; NM_130458); MMP-9 (nt. 1937–2258; NM_008610); VEGF (nt. 115–539; gi/249858); *Ihh* (nt. 897–1954; MN_010544); *Shh* (full coding sequence; NM_009170); *Patched-1* (nt. 81–841; NM_008957); *Smoothened* (nt. 450–1000; BC048091); collagen X (nt. 1302–1816; NM009925); and collagen II (nt. 1095–1344; X57982). After hybridization, slides were washed with 2× SSC containing 50% formamide at 50°C, treated with 20 μg/ml RNase A for 30 min at 37°C, and washed three times with 0.1× SSC at 50°C for 10 min/wash. Sections were dehydrated with 70, 90, and 100% ethanol for 5 min/step, coated with Kodak NTB-3 emulsion diluted 1:1 with water, and exposed for 10 to 14 days. Slides were developed with Kodak D-19 at 20°C and stained with hematoxylin. Dark and bright field images were captured using a digital camera, and dark field images were pseudo-colored using Adobe Photoshop software.

For in situ hybridization using DIG labeled probes, paraffin serial sections were pretreated with 0.2 N HCl for 10 min, 0.25% acetic anhydride in triethanolamine buffer for 15 min, and 1 μg/ml proteinase K in 50 mM Tris–HCl, 5 mM EDTA pH 7.5 for 1 min at room temperature and then immediately post-fixed in 4% paraformaldehyde for 10 min. Sections were hybridized with antisense or sense DIG-probes (approximately 0.5 μg/ml) at 55°C for 16 h. Slides were washed with 2× SSC containing 50% formamide at 55°C for 30 min, three times with TN (10 mM Tris–HCl pH 7.5; 0.5 M NaCl), treated with 20 μg/ml RNase A for 30 min at 37°C, washed two times with 1× SSC containing 25% formamide at 55°C for 30 min/wash, and finally stained in NBT/BCIP color substrate solution.

Chondrocyte cultures and proteoglycan analyses

Synchondroses were isolated microsurgically from E17.5 wild-type and Ihh embryos under a dissecting microscope, making sure they were devoid of adhering tissues as much as possible. Tissue fragments were incubated for 30 min at 37°C in 1× trypsin/EDTA mixture (Gibco) in Ca/Mg-free Hank's saline containing 60 μg/ml kanamycin and 1×Fungizone (Gibco) with gentle vortexing every 10 min. Released cells were discarded, and remaining tissue fragments were further incubated for 1.5 h in serum-free DMEM containing 1.7 U/ml of type I collagenase (Worthington) at 37°C. After removal of enzyme mixture, tissue fragments were dissociated into a single cell suspension by gentle pipetting and cells were plated at an initial density of 2×10^4/well in type I collagen-coated 48 multi-well tissue culture plates. Medium was HG-DMEM containing 10% fetal bovine serum (Hyclone) and 10 μg/ml ascorbic acid. On days 4–5 of culture, the subconfluent cells were given fresh medium containing appropriate additives, including 100 ng/ml rhBMP-2 (Yamanouchi Phar. Co.), 1 μg/ml rhIHH (R&D) and/or 0.3 μg/ml Noggin (R&D). Medium was changed every other day, and fresh additives were provided.

To monitor proteoglycan accumulation, cultures were fixed with 3% acetic acid pH 1.0 for 5 min and stained with 0.1% Alcian blue in 3% acetic acid pH 1.0 (Enomoto-Iwamoto et al., 2000). After exhaustive rinsing, cell layer-associated Alcian blue was solubilized in DMSO and quantified spectrophotometrically. DNA content was measured (Johnson-Wint and Wellis, 1982) and used for normalization.

Results

Deranged synchondrosis development in $Ihh^{-/-}$ mice

Skulls from wild-type and $Ihh^{-/-}$ E15.5 and E17.5 mouse embryos and newborn (P0) mice were stained with Alcian blue to depict cartilaginous structures. Wild-type cranial bases displayed their expected staining features and an elongated shape along the antero-posterior axis. The central portion was uniformly cartilaginous and Alcian blue-positive at E15.5