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Confocal Laser Scanning Microscopic Analysis of Collagen Scaffolding Patterns in Cranial Sutures

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Abstract

Although recent studies indicate that regional dura mater influences the fate of the overlying cranial suture, little is known about the assembly of extracellular matrix (ECM) molecules within the patent and fusing murine cranial suture complexes. Confocal laser scanning microscopy was used to study ECM assembly within patent and fusing cranial suture complexes. Coronal sections (20 μ m thick) of patent sagittal (SAG) and fusing posterior frontal (PF) sutures from postnatal 8-, 14-, and 18-day-old Sprague-Dawley rats were scanned in 0.5- μ m increments, and images were collected consecutively to create a z-series for three-dimensional reconstruction. Spatial and temporal collagen arrangements were compared between SAG and PF sutures by measuring interfiber distance, fiber thickness, and total collagen surface area at each time point.

We demonstrate that on day 8 (before the onset of suture fusion), collagen bundles are randomly arranged in both the SAG and PF sutures. By day 14 (midfusion period), there was a statistically significant reduction in total collagen surface area (80.5% versus 67.4%; $P < 0.05$) as the collagen bundles were organized into orthogonal lattices along the anterior and endocranial margins of the PF suture. Furthermore, new bone matrix deposition was observed along the edges of these organized collagen bundles. In contrast, collagen within the SAG suture remained randomly arranged and unossified. By day 18 (late fusion period), the PF suture was completely fused except for the posterior-ectocranial portion. This patent section of the PF suture contained a highly organized mineralizing orthogonal collagen lattice. The total collagen surface area in the day-18 PF suture continued to decline compared with the day-8 PF suture (80.5% versus 55.6%; $P < 0.05$). In the day-18 SAG suture, the collagen bundles remained randomly arranged, and the total surface area did not change. The same analysis was performed in a human pathologic fusing and patent suture. Similar results were observed. The total collagen surface area significantly decreased in the pathologic fusing human suture compared with the patent suture (92.8% versus 60.6%; $P < 0.05$). Moreover, the pathologically fusing suture contained a highly organized mineralizing orthogonal collagen lattice. This is the first analysis of collagen patterns in patent and fusing cranial sutures.

Keywords

Cranial suture biology; collagen; craniosynostosis; confocal scanning laser microscopy

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Craniosynostosis occurs in approximately 1:2000 live births.¹ Although nonsyndromic synostoses are more common, more than 150 genetic syndromes have been identified.² Recent advances in genetic mapping have linked chromosomal mutations with craniosynostotic syndromes.³ Despite the identification of these genetic mutations, the fundamental biomolecular mechanisms mediating human cranial suture biology remain unknown. Today, many laboratories are investigating murine cranial suture biology as a model for human cranial suture development and fusion. Normal murine cranial suture biology is complex, but evidence suggests that the dura mater provides the biomolecular blueprints (e.g., the soluble growth factors), which guide the fate of the pluri-potent osteogenic fronts.⁴ Although our knowledge of these dura-derived signals has increased dramatically, we have barely begun to understand the fundamental mechanisms that mediate cranial suture patency or fusion.

Although recent studies indicate that regional dura mater influences the fate of the overlying cranial suture, little is known about the assembly of extracellular matrix (ECM) molecules within the patent and fusing murine cranial suture complexes.⁴ Extracellular matrix is composed of structural proteins, anchor proteins, and proteoglycans. Cellular adhesion, migration, proliferation, and differentiation are all influenced by the composition and structure of the surrounding ECM.⁵ While studies have investigated the production of ECM products in calvarial osteoblasts derived from patent and fusing cranial sutures, to date, the three-dimensional structure of the ECM in patent and fusing cranial suture remains unknown.⁴

Cell-ECM adhesion is crucial for control of cell behavior.⁶ The ECM is connected to the intracellular cytoskeleton through transmembrane proteins (e.g., integrins) and several dozen membrane-proximal proteins. These proteins transduce bidirectional signals between the extracellular and intracellular compartments. The subcellular machinery that mediates cell-ECM adhesion and signaling is complex. Furthermore, despite sharing common components, cell-ECM adhesion can result in considerably desperate responses in different cell types. Because collagen is the primary ECM scaffold of bone, we used confocal laser scan microscopy on planar tissue sections of an adjustable thickness to perform a three-dimensional tissue image reconstruction. We focused this first study of cranial suture matrix on collagen because its heterotrimeric helix is reinforced with calcium hydroxyapatite providing infrastructural support and tensile strength to the cranium.

We hypothesize that, in conjunction with dura mater-derived cytokines, the temporal and spatial changes in suture mesenchyme collagen expression and fibril orientation, in the fusing sutures, facilitate the formation of early bone spicules extending from the osteogenic fronts toward the center of the suture mesenchyme, resulting in physiologic or pathologic cranial suture fusion.

Materials and Methods

Animals

All experiments were performed in accordance with the New York University Animal Care and Use Committee. Adult male rats weighing 250 to 350 g were purchased from Tachonic Laboratories (Germantown, NY). Animals were housed individually in a light- and temperature-controlled environment. Animals were given food and water ad libitum. Eighteen animals were divided into 3 groups according to age: postnatal days 8, 14, and 18 (N = 6 in each age group). Animals were killed with an intraperitoneal injection of 0.10 mL pentobarbital 1 hour after infusion of fluorescent and radiolabeled annexin V as outlined below. Strip craniectomies including a 1.0 × 0.6-cm sample including the posterior frontal (PF), sagittal (SAG), and bilateral coronal sutures were harvested and saved for subsequent image analyses. Care was taken to dissect the calvaria from all surrounding nonosseous tissue including the periosteum and underlying dura mater.

Human Cranial Sutures

Patent and fused SAG sutures were obtained from an infant (11 months of age) with nonfamilial, nonsyndromic sagittal synostosis. The specimens were frozen in liquid and stored at -70°C. Human specimens were prepared and analyzed in parallel with rat cranial sutures.

Tissue Harvest and Processing

Cranial sutures were fixed in 4% paraformaldehyde overnight, dehydrated through graded ethanols, embedded in paraffin, and cut into 7- μ m serial sections for hematoxylin and eosin staining and immunohistochemical and confocal microscopic analysis. Suture location was identified grossly during embedding and serial sectioning by the visible permanent dye in the tissue sample.

Hematoxylin and Eosin Staining

Sutures mounted on Fisher Superfrost slides (Fisher Scientific, Pittsburgh, PA) were stained with hematoxylin and eosin using a standard protocol and viewed with a Leica-DMLB microscope (Leica Microsystems Inc).

Confocal Laser Scanning Microscopy

Collagen architecture was analyzed using confocal laser scanning microscopy. Cranial suture sections were stained by the modified picosirius red technique described by Dolber and Spach.⁷ Briefly, sections of cranial sutures were deparaffinized in xylenes and rehydrated with 100%, 95%, and 70% ethanol and distilled water. After 1 minute in 0.2% aqueous phosphomolybdic acid to render the cytoplasm colorless, sections were stained in a 0.1% solution of Sirius red F3BA (CT no. 35780; Pfaltz and Bauer, Stamford, CT) in saturated aqueous picric acid for 90 minutes. Sections were then washed in 0.01 N HCl for 2 minutes, dehydrated, cleaned, and mounted. All sections were examined by using a Carl Zeiss LSM 310 laser scanning confocal microscope (Thornwood, NY) that was equipped with 2 lasers—an external argon ion blue laser (488/514 nm) for excitation of green fluorophores and a helium-neon green laser (543 nm) for excitation of red fluorophores. The cranial sutures were first identified with a Plan-Neofluar 20 $\times$ multi-immersions objective (numerical aperture, 0.75) by conventional light. The system was then switched to the frame mode, and confocal pictures were generated with the 543-nm line of helium-neon laser (attenuation = 1) with Plan-Neofluar 20 $\times$, 40 $\times$, and 63 $\times$, and 100 $\times$ oil immersion objectives for the fluorescent imaging. The emission light beam was recorded by a photomultiplier after passing a pinhole aperture and emission filter. The depth of the optical section (confocal z resolution) was kept constant by holding the size of the aperture pinhole constant in the emission pathway. The scanning time setting for image collection was 8 seconds, with the average set to reduce photon noise. The bandwidth filter was set to 1 for the sharpest images. Sections were scanned and collected with 0.5- μ m increments to the entire section thickness of 7 μ m. Digital data of frame-mode images were recorded and stored as 512 $\times$ 512 pixel images.

To evaluate interfiber distances, unaltered images of fused and patent sutures at the same magnification were used. Interfiber distances were measured in the vertical direction with Photoshop 5.0 software (Adobe, San Jose, CA). A total of 3 samples were used for each time point. Each image was divided into 4 quadrants, and there were 10 measurements per quadrant. Therefore, 40 measurements were made on each sample. Mean values for interfiber distance were determined, and total collagen surface area was calculated. Student's *t*-test was used for statistical analysis of the mean interfiber distance, fiber thickness, and total surface area from the 3 patent and 3 fusing suture sections per time point. A *P* value of less than 0.05 was considered significant.

Results

Prefusion Period (Day 8)

We demonstrate that on day 8 (before the onset of suture fusion), collagen bundles are randomly arranged in both the PF and SAG sutures. The average interfiber distance was 27 μm in the PF suture and 22 μm in the SAG suture ($P < 0.05$; Table 1). The average total collagen surface area was 80.5% in the PF suture and 72.5% in the SAG suture ($P > 0.05$; Table 1).

Active-Fusion Period (Day 14)

By day 14 (midfusion period), collagen bundles are organized into an orthogonal lattice along the anterior and endocranial margins of the PF suture (Fig 1). Furthermore, new bone matrix deposition was observed along the edges of these organized collagen bundles. In contrast, collagen within the SAG suture remained randomly arranged and unossified. The average interfiber distance was 22 μm in the PF suture and 17 μm in the SAG suture ($P < 0.05$; Table 1). The average total collagen surface area in the PF suture significantly decreased compared with the day-8 PF suture as the collagen bundles became more organized (80.5% versus 67.4%). The average total collagen surface area in the SAG suture did not change compared with the day-8 SAG suture (72.5% versus 62.7 %; $P > 0.05$).

End-of-Fusion Period (Day 18)

By day 18 (late fusion period), the PF suture was completely fused except for the posterior-ectocranial portion (Fig 1). This patent section of the fusing PF suture contained a highly organized mineralizing orthogonal collagen lattice. In contrast, the day-18 SAG suture collagen remained randomly arranged. The average interfiber distance was 45 μm in the PF suture and 23 μm in the SAG suture (45 μm versus 23 μm ; $P < 0.05$; Table 1). The average total collagen surface area in the PF suture continued to decrease compared with the day-8 PF suture (80.5% versus 55.6%; $P < 0.05$). There was no significant change in the average total collagen surface area in the SAG suture (71.5% versus 72.5%; $P > 0.05$; Table 1).

Patent and Fusing Human Cranial Sutures

Multiple sections from patent and fused portions of the sagittal suture of an 11-month-old child with nonfamilial, nonsyndromic sagittal synostosis were analyzed (Fig 2). The average interfiber distance was 23 μm in the fusing portion of the suture and 17 μm in the patent portion of the suture ($P < 0.05$; Table 1). Moreover, the average total collagen surface area was significantly reduced in the pathologically fusing portion of the SAG suture compared with the patent portion of the SAG suture (60.6% versus 92.8%, $P < 0.05$; Table 1).

Discussion

Four etiopathogenic theories have dominated the study of craniosynostosis. In 1851, Rudolph Virchow⁸ suggested that the fusion of cranial sutures is independent of the neurocranial environment. In 1920, Park and Powers⁹ theorized that a defect in the cranial suture mesenchyme leads to premature suture fusion. In 1959, Moss¹⁰ postulated that tension from an abnormally formed skull base was transmitted through the dura mater affecting the fate of the overlying cranial suture. More recently, a convincing body of experimental evidence suggests that the regional cranial suture-associated dura mater is responsible for determining the fate of the overlying cranial suture. These studies have demonstrated that cytokines can affect cellular activity and control suture fate; however, few have investigated the role of the ECM on suture fate.¹¹ Extracellular matrix components contain signaling domains that can activate cell surface receptors and induce proliferation, migration, differentiation, and apoptosis.¹² Unlike traditional growth factors, these “matrikines” possess relatively low

binding affinity (high nanomolar or micromolar) and are often present in multiple valency.¹³ Extracellular matrix organization may affect matrikine activity and, in turn, affect cell and cranial suture fate. To date, the three-dimensional structure of the ECM in patent and fusing cranial sutures remains unknown. This study was designed to determine if there was a difference in the three-dimensional structure of the ECM in patent and fusing cranial sutures.

To explore the relationship between the ECM and suture fusion, we used confocal microscopy to demonstrate a progressive reduction in total collagen surface area as the collagen bundles in the fusing PF suture became organized into an orthogonal lattice. Moreover, new bone matrix deposition was observed in these organized collagen bundles. In marked contrast, collagen bundles in the patent SAG sutures remained randomly arranged. This lack of collagen organization in the SAG suture may work in concert with antiosteogenic factors (e.g., Noggin) to maintain suture patency by reducing the osteogenic infrastructure at the center of the cranial suture mesenchyme.¹⁴ Interestingly, we found similar patterns of collagen organization and arrangement in patent and pathologically fusing portions of a human SAG suture. We hypothesize that collagen organization and architecture, in the fusing suture, facilitate the formation of bone spicules extending from the osteogenic front toward the center of the intercalary suture mesenchyme. Furthermore, we suspect that this process is mediated by bidirectional signaling between the ECM and intracellular compartment through transmembrane proteins and cytokines (Fig 3).

Collagen is the primary ECM protein found in vertebrates. Not only does the heterotrimeric collagen helix provide infrastructural support and tensile strength, but it is also an important regulator of cell function.¹⁵ The equilibrium between the synthesis and degradation as well as the organization of collagen fibrils and fibers is therefore critical to physiologic processes. For example, an imbalance in collagen fibril formation and degradation can lead to pathological conditions such as fibrosis.¹⁶ One group of receptors that interpret the composition/organization of collagens in the ECM are the discoid domain receptors (DDR).¹⁶ Binding of collagen to DDRs inhibits collagen fibrillogenesis and alters the morphology of collagen fibers; this is likely mediated by the upregulation of matrix metalloproteinases.¹⁷ We have previously found DDR expression on calvarial osteoblasts (Smith LP, 2001, unpublished observation). It is possible that DDRs may play a role in interpreting the collagen in the suture mesenchyme of a patent or fusing suture and, in turn, affecting suture fate (Fig 3).

Cell function is also mediated through transmembrane integrins interactions with the ECM.⁶ The subcellular machinery that mediates cell-ECM adhesion and signaling is complex. These signals can activate growth-promoting pathways such as the Ras-ERK cascade and the immediate-early gene c-FOS or signal through the JNK pathway to regulate the progression through the cell cycle.¹⁸ Furthermore, loss of attachment to the ECM causes apoptosis in many cell types via the FAK-Rb pathway.¹⁹

Here we show that collagen bundles became organized into an orthogonal lattice in the fusing PF suture, whereas the collagen remains randomly arranged in the patent SAG suture. We demonstrate the same pattern of collagen architecture in cranial sutures obtained from a human infant. Furthermore, we find that the collagen architecture in the SAG suture is similar to the architecture observed in the normal patent sutures of infants. We propose that the collagen architecture in the murine PF suture is similar to the architecture observed in prematurely fusing sutures. We hypothesize that the ECMs, via integrins and DDRs, play an important role in maintaining the balance between suture fusion and patency. In craniosynostosis, we predict that excessive integrin signaling or inadequate DDR signaling may be responsible for premature organization of collagen, bone formation, and suture fusion. In conjunction with previously published cytokine expression profiles in fusing sutures, we hypothesize that the temporal and spatial changes in collagen expression and fibril orientation facilitate the

formation of early bone spicules extending from the osteogenic front toward the center of the suture mesenchyme, which leads to premature bone formation and suture fusion.

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Fig 1. Rat cranial sutures. To explore the relationship between the ECM and suture fusion, our laboratory uses the rat model of suture fusion. Using confocal microscopy, we demonstrated progressive increase in interfiber distance and reduction in total collagen surface area as the collagen bundles became organized into an orthogonal lattice in the fusing PF suture (right). In marked contrast, collagen bundles in the patent SAG sutures remained randomly arranged at all time points (left).

Fig 2.

Human cranial sutures. We demonstrate increased interfiber distance and reduced total collagen surface area as the collagen bundles became organized into an orthogonal lattice in the fusing cranial sutures obtained from human infants (right). As in the rat model, human collagen bundles in the patent sutures remained randomly arranged (left). We propose that the collagen architecture in the PF suture models, the architecture observed in craniosynostosis; and the collagen architecture in the SAG suture models, the architecture observed in the normal patent sutures of human infants.

Fig 3.

Major signaling pathways of cranial suture fusion. Discoid domain receptors (DDR) and integrins are 2 groups of proteins that regulate the compositions of collagens in the ECM. It is thought that the binding of collagen to DDRs inhibits collagen fibrillogenesis through the upregulation of matrix metalloproteinases (MMPs). The integrins activate growth-promoting signaling pathways such as the Ras, ERK, and c-FOS cascade, signal through the JNK pathway to regulate the progression through the cell cycle, and inhibit apoptosis. We speculate that these pathways are involved in cranial suture biology.

Table 1
Collagen Interfiber Distances and Total Surface Area in Patent and Fusing Rat and Human Cranial Sutures

<i>Cranial Sutures</i>	<i>Interviber Distances, μm</i>	<i>Total Surface Area, %</i>
SAG		
Day 8	22	72.5
Day 14	17	62.7
Day 18	23	71.5
PF		
Day 8	27 [*]	80.5
Day 14	22 [*]	67.4 [†]
Day 18	45 [*]	55.6 [†]
Human		
Patent	17	92.8
Fusing	232	60.6 [‡]

^{*} $P < 0.05$ compared with corresponding days in the sagittal suture.

[†] $P < 0.05$ compared with posterior frontal on day 8.

[‡] $P < 0.05$ compared with human patent suture.