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Tissue interactions between craniosynostotic dura mater and bone

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Abstract

Background—Cells within the dura mater have been implicated in the determination of suture patency and fusion. Craniosynostosis (CS), the premature fusion of one or more of the cranial sutures, could result from abnormal control over the differentiation of osteoprogenitor cells from the dura mater. This study tested whether dura mater cells derived from rabbits with congenital CS were different from cells derived from normal rabbits and investigated the effects that CS dura mater had on osteogenic differentiation *in vitro* and *in vivo*.

Methods—Cells were derived from the dura mater from wild-type rabbits (WT; n=23) or CS rabbits (n=16). Cells were stimulated with bone morphogenetic protein 4 (BMP4) and alkaline phosphatase (ALP) expression and cell proliferation were assessed. Dura mater-derived cells were also co-cultured with primary rabbit bone-derived cells and ALP was assessed. Finally, interactions between the dura mater and overlying tissues were manipulated *in vivo*.

Results—CS dura mater-derived cells proliferated faster than WT cells, but were not more ALP positive. Co-culture experiments showed that CS dura mater cells induced increased ALP activity in CS bone-derived cells, but not in WT bone-derived cells. *In vivo* experiments showed that a physical barrier successfully inhibited dura mater-derived osteogenesis.

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All authors have no conflicts of interest.

Conclusions—Co-culture of CS bone- and CS dura mater-derived cells evoked an abnormal phenotype *in vitro*. Covering the CS dura mater led to decreased bone formation *in vivo*. Further investigations will focus on the signaling molecules involved in the communication between these two CS tissues types *in vitro* and *in vivo*.

Keywords

craniosynostosis; rabbit; osteogenic differentiation; dura mater; *in vivo*

Introduction

The premature fusion of one or more of the calvarial sutures has been termed craniosynostosis (CS). CS results from abnormal regulation of bone formation; specifically, bone forms within the calvarial suture. Our understanding of the molecular and cellular microenvironments involved in suture formation and fusion is improving. Complex interactions among growth factors have been implicated in the formation, maintenance, and fusion of the calvarial sutures^{1–10}. These molecular (growth factor) interactions influence tissue function within or around the suture. Alterations in the biology of the cellular component of the suture may result in tissue dysfunction. A broader understanding of the control of bone formation at the suture requires comprehensive analysis of the cellular components of the suture and the interactions among these cellular components. In this regard, characterization of the cells within and around areas of bony overgrowth (fused sutures and perisutural tissues) may 1) give insight into the mechanisms that cause craniosynostosis and 2) allow for development of more effective management modalities. Furthermore, an in depth understanding of the communication among tissues within and around the suture may illuminate therapeutic design.

The dura mater is the tissue that exists between the calvarial bones and the brain and consists of fibrous and osteogenic layers. Previous investigations have implicated the dura mater in the regulation of suture fusion^{11–15}. The results of these studies suggest that the dura mater underlying a suture expresses soluble proteins that function to either maintain suture patency or induce suture fusion. Specifically, the data suggest that there are regional differences in the dura mater that mediate suture patency, meaning the dura mater under the suture may have different protein expression or different molecular regulation compared to areas not directly contacting a patent suture^{14–19}. However, these studies were performed on rodent models of normal programmed fusion (in the case of the mouse and rat interfrontal suture)^{9,13–15,17} or experimentally induced (in the case of the rat coronal suture organ culture)^{4,11,20–22}. The results of those experiments are confounded by the lack of a craniosynostosis model and by the lack of appropriate unaffected controls. Furthermore, no studies have investigated the interactions between tissues derived from animals with naturally occurring craniosynostosis.

Increased bone formation leading to suture fusion could result from dysregulation of the osteogenic differentiation of osteoprogenitor cells in the perisutural region. The dura mater is thought to be one source of osteoprogenitors in the calvaria^{23–27}. Increased osteogenesis may result from either increased numbers of osteogenic cells or from increased osteogenic response to normal stimuli. Finally, the cells of the dura mater may express abnormal types or amounts of soluble signaling proteins that can lead to craniosynostosis.

We have previously identified and cultivated a colony of New Zealand White rabbits with familial congenital craniosynostosis²⁸ that can be used to address some of the limitations of other research models^{29–32}. Using this model, we designed the current study to test the hypothesis that cells derived from the dura mater of craniosynostotic rabbits would be

different compared to cells derived from the dura mater of wild-type (WT) rabbits. Specifically, we thought that the CS dura mater cells would either proliferate faster or react more strongly to osteogenic stimulation. To test this hypothesis, we isolated primary cells from the dura mater of CS and WT rabbits and from the calvarial bones of these two groups. Dura mater-derived cells were subjected to osteogenic stimulation using recombinant human bone morphogenetic protein 4 (rhBMP4) or osteogenic medium, and bone-derived cells were cultured in the presence (co-culture) of either CS or WT dura mater cells.

After identifying that the molecular communication between craniosynostotic dura mater cells and craniosynostotic bone cells was altered, we secondarily sought to test the hypothesis that calvarial suture maintenance can be achieved by physically blocking the communication between the craniosynostotic dura mater and the overlying tissue.

Materials and Methods

Tissue Harvest

Calvarial bone tissue and associated underlying dura mater were harvested from wild-type (n=23) rabbits and rabbits from the breeding colony with coronal suture synostosis (n=16) housed at the University of Pittsburgh under an IACUC approved protocol as previously described³³. A craniosynostosis diagnosis resulted from the absence of a patent coronal suture at 10 days of age. Following diagnosis, the region of the skull (approximately 5mm × 5mm) that contained the fused coronal suture (synostotic suture bone) or the normal patent coronal suture (wild-type suture bone) was removed using a small periosteal elevator and a 0.5mm dental bur, fixed to a low-speed hand engine. Care was taken to preserve the underlying dura mater while removing the bone. A small (approximately 5mm × 5mm) region of the dura mater underlying the removed bone was also harvested. From the same animals, a small region, approximately 5mm × 5mm square, of the center of the parietal bone, not associated with any suture (non-suture bone) was removed in a similar manner. Non-suture associated dura mater samples were also collected from each animal.

Excised bone samples were cut into small pieces and placed into ice cold phosphate buffered saline (PBS) until harvest was concluded. The dura mater was left intact and placed in ice cold PBS. Each animal's tissues were kept separate for cell isolation and subsequent culture, meaning that samples were not pooled.

Cell Isolation

Bone and dura cells were released through a 30 minute digestion in a 0.1% collagenase I (Sigma) solution in Hank's balanced salt solution. The digests were centrifuged and the pellets and undigested tissue pieces were resuspended in proliferation medium (PM; Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [Atlanta Biologicals] and 1% penicillin/streptomycin [Invitrogen]). The cell suspensions and undigested tissue pieces were plated and cultured until cells were identified surrounding tissue pieces, similar to previously published work³⁴. Cells were trypsinized and replated after the remaining tissue pieces were removed. Cell suspensions were cultured by feeding every 2–3 days and passaging as needed. All cells were used before the 4th passage.

Cell Proliferation

The effects of rhBMP4 (R&D Systems) stimulation on dura cell proliferation were assessed via CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega, cat #G3581). Cells were seeded in triplicate into 96-well plates at a density of approximately 1,000 cells/well and treated with PM only, PM supplemented with 50ng/ml rhBMP4, or with osteogenic medium (OM; DMEM supplemented with 10% fetal bovine serum [CellGro], 1% penicillin/

streptomycin [Invitrogen], 50µg/ml Absorbic-Acid-2-Phosphate, 10mM β-glycerophosphate, and 0.1µM Dexamethasone) for 3 or 7 days. After 3 or 7 days of treatment, cells were incubated for one hour with 20µl/well of CellTiter96 Aqueous One solution according to manufacturer's instructions. The absorbance at 490nm was recorded with a 96-well plate reader (Benchmark Plus, BioRad).

Alkaline Phosphatase Assays

To determine dura cell differentiation potential via rhBMP4 stimulation, cells were seeded in triplicate onto 24-well plates at a density of approximately 5,000 cells/well. Cells were treated with PM or PM with 50ng/ml of rhBMP4 for either 3 or 7 days at which point, they were stained for alkaline phosphatase presence (ALP, an early marker of osteogenic differentiation) or lysed for quantitative ALP (QALP) analysis. Qualitative ALP staining was determined according to the manufacturer's protocol (Sigma-Aldrich, cat #86C-1KT). Briefly, cells were fixed with 3.7% formaldehyde, washed 3 times with deionized water, stained for 30 minutes, and then assessed qualitatively with light microscopy. QALP activity was measured via absorbance of cell lysate after treatment with SIGMA FAST p-Nitrophenyl Phosphate (Sigma, cat #N2770). Measures were normalized to amount of total protein per volume of lysate. Total protein was determined in identical volumes of cell lysate with a BCA Protein Assay (Pierce, cat #23227). Normalized QALP activity was calculated with the following formula: QALP activity (µmol pNPP/ml) / protein (µg/ml) × 1000µg/mg × 1000nmol/1µmol = nmol pNPP/mg protein. All measures were completed in triplicate wells for each animal.

Co-Culture

To determine the effect that dura mater-derived cells might have on bone-derived cells, they were cultured together. Bone-derived cells were seeded in triplicate onto 24-well plates at a density of approximately 5,000 cells/well. At the same time dura mater-derived cells were seeded at the same density on transwell membrane co-culture baskets (Corning 3470) with a pore size of 0.4µm. After 24 hours, transwell baskets were transferred to the bone-seeded 24 well plates to initiate co-culture. Cells were fed every other day with either PM or PM supplemented with 50ng/ml BMP4. After 7 days of co-culture, the transwell baskets were discarded and the bone-derived cells were assessed for qualitative and quantitative ALP activity via the above protocols.

In vivo transplant experiments

Data were collected from 114 rabbits: 72 normal controls and 42 rabbits with unicoronal suture synostosis (17 unoperated controls; 6 surgical controls; 6 suture only; 6 barrier only; and 7 suture and barrier). All rabbits were obtained from a breeding colony of rabbits with familial craniosynostosis that was developed and maintained at the University of Pittsburgh. At 10 days of age all rabbits had amalgam markers placed on either side of the frontonasal, coronal, and anterior lambdoidal sutures for sutural growth assessment as previously published^{28,31,35}. At 25 days of age, 25 synostosed rabbits had a 3–5mm long unicoronal suturectomy (Figure 1). Coronal suture autografts (3–5mm long) were harvested from the middle third of the patent side in all rabbits and transplanted with or without impermeable, polytetrafluoroethylene (PTFE, gore-tex) membranes over the dura mater on the synostosed side (Figure 1). At 84 days of age, the coronal suturectomy sites were harvested for histological analysis. The sutures were fixed in formalin, paraffin embedded, and sectioned in the sagittal plane at a thickness of 5µm for qualitative hematoxylin and eosin staining.

Statistical Analyses

For cell differentiation and proliferation, either a $2 \times 2 \times 3 \times 2$ four-way (for QALP) or a $2 \times 2 \times 3$ three-way (for proliferation) Analysis of Variance was conducted for cell type (WT, CS), bone region (suture bone, non-suture bone), dose (0 or 50 ng/ml of rhBMP4), and time of treatment (3 days or 7 days for QALP data). Intergroup differences were assessed using a post-hoc Bonferroni multiple comparison test. Mean differences were considered significant if $p < 0.05$.

To assess whether there was a difference in the frequency of positive scores for matrix mineralization, a Fisher's Exact test was utilized comparing wild type and synostotic groups by suture and non-suture bone at 7 days, 14 days, and 21 days of treatment. Differences were considered significant if $p < 0.05$.

Results

Dura mater-derived cells

Proliferation—Dura cell proliferation was assessed using a four way ANOVA. Subject variables in the analysis were dura type (WT versus CS), dura area (suture associated or non-suture associated), time in culture (3 or 7 day assay), and treatment (PM, PM with BMP4 50 ng/ml, or OM). The assumptions of normality and homogeneity of variance were met. Four way and three way interactions were not significant, $p > 0.05$. For the two way interactions only time by type ($F=24.888$, $p < 0.001$) and type by treatment ($F=7.587$, $p=0.001$) were significant. Proliferation was greatly increased in CS cells between 3 and 7 days (3 day: $x=0.4585$, $se=0.0188$; 7 day: $x=0.8885$, $se=0.0428$) compared to the wild type cells (3 day: $x=0.4467$, $se=0.0139$; 7 day: $x=0.5804$, $se=0.0213$; Figure 2). The CS dura mater cells (PM: $x=0.6215$, $se=0.0408$; BMP4: $x=0.6436$, $se=0.0473$; OM: $x=0.9376$, $se=0.0769$) responded to all treatments with greater proliferation than the WT cells (PM: $x=0.4840$, $se=0.0182$; BMP4: $x=0.5079$, $se=0.0180$; OM: $x=0.6700$, $se=0.0611$). However, CS cells show little difference between control and BMP4-treated conditions and show much greater proliferative response to osteogenic medium. The dura area (suture vs. non-suture) main effect was not significant, $p > 0.05$. Treatment main effect was significant, $F=20.214$, $p < 0.05$. Post-hoc Bonferroni analysis showed OM ($x=0.8430$, $sem=0.0569$) to induce greater proliferation than either PM ($x=0.5398$, $sem=0.0207$) or BMP4 ($x=0.5629$, $sem=0.0228$), $p < 0.001$ (Figure 2). The time main effect was significant, $F=123.423$, $p < 0.001$, with the 7 day assays ($x=0.7282$, $sem=0.0265$) showing greater proliferation than 3 day ($x=0.4515$, $sem=0.0112$). The dura type main effect was significant, $F=23.702$, $p < 0.001$, with the CS cells ($x=0.7108$, $sem=0.0326$) showing greater proliferation than WT ($x=0.5158$, $sem=0.0140$).

ALP activity—Mean differences in ALP activity in cultured cells derived from the dura mater were analyzed using a four way ANOVA. Subject variables in the analysis were dura type (WT versus CS), dura area (suture associated or non-suture associated), time in culture (3 or 7 day assay), and dose (0 or 50 ng/ml BMP4). A natural log transformation allowed for the assumption of normality and homogeneity of variance to be met. Four way, three way, and two way interaction terms were not significant, $p > 0.05$, with the exception of dura type by dura area, $F=5.73$, $p=0.018$. WT suture dura appeared to have higher ALP ($x=47.98$, $se=8.51$; Figure 3) than non-suture dura ($x=23.54$, $se=3.19$). The craniosynostotic relationship is the opposite with the non-suture dura ($x=17.49$, $sem=2.65$) having slightly greater differentiation than the suture dura ($x=10.15$, $sem=1.02$). The main effects of time, dura area, and dose were not significant, $p > 0.05$; however, the main effect of dura type was significant, $F=6.44$, $p=0.012$, with the WT dura ($x=33.38$, $sem=3.99$) exhibiting greater differentiation than craniosynostotic ($x=14.56$, $sem=1.70$).

Co-cultured bone-derived cells

ALP activity—Mean ALP activity of bone cells co-cultured with dura was compared using a three way ANOVA. Subject variables used in the analysis were bone type (WT versus CS), culture condition (no co-culture, co-culture with WT dura, and co-culture with CS dura), and dose (0 or 50 ng/ml BMP4). A natural log transformation allowed for both the assumptions of normality and homogeneity of variance to be met. The three way interaction term and the two way interaction terms of dose by culture condition and bone type by dose were not significant, $p > 0.05$. Bone type by culture condition was significant $F = 10.470$, $p < 0.001$. The CS bone with CS dura co-culture had the greatest values for differentiation (Figure 4). The culture condition main effect was not significant, $p > 0.05$. The bone type main effect was significant, $F = 4.905$, $p = 0.027$ with greater mean values for CS derived bone ($x = 83.8018$, $sem = 9.2243$) compared to WT (67.3039 , $sem = 16.8368$). The dose main effect was significant, $F = 49.170$, $p < 0.001$, with the BMP4 treatment having greater mean values ($x = 109.9611$, $sem = 16.8368$) than those cells cultured in PM ($x = 46.4582$, $sem = 5.8758$).

In vivo suture transplant

Histological examination revealed that rabbits with suturectomy and suture only grafts showed extensively reossified and resynostosed suturectomy sites and autografts while rabbits with barriers showed patent suturectomy sites or autografts superficial to the membrane (Figure 5). Both groups with dural barriers also showed bone formation deep to the barrier (Figure 5).

Discussion

The current study was undertaken to test the hypothesis that cells derived from the dura mater of craniostomotic rabbits would be different from cells derived from the dura mater of wild-type rabbits. Because the dura mater has been shown to play an important role in regulating suture patency^{11–15}, cells derived from animals with abnormally fusing sutures should show phenotypic differences compared to wild-type cells. Therefore, we isolated primary cells from the dura mater of WT rabbits and from rabbits with CS and assessed cell proliferation and ALP activity.

A standard number of cells were seeded in each well, cells were cultured in different media, and total cell number was estimated using a colorimetric assay. CS dura mater-derived cells were found to proliferate faster than WT dura mater cells. Faster proliferation of dura mater cells may increase the pool of osteoprogenitor cells in the suture region, thereby increasing the overall bone formation, consistent with the hypothesis that the dura mater may be involved in CS in this model. The different media tested (PM, PM containing BMP4, and osteogenic medium [OM]) affected WT and CS cells similarly, with OM inducing the highest proliferation in both WT and CS cells, suggesting that CS cells had an intrinsic increased proliferation rate and not an increased reaction to a specific stimulation.

ALP activity can be used as an early marker of osteogenic differentiation. We anticipated that CS dura mater-derived cells would demonstrate higher levels of ALP activity compared to WT controls. Interestingly, this did not seem to be the case. WT dura mater-derived cells showed higher ALP activity across treatments, mostly due to the increased ALP in cells derived from dura mater underlying a patent WT coronal suture. Although these results were unexpected, they can be explained considering the WT patent suture is the only active growth site being tested in this study. The WT non-suture associated dura mater, the CS non-suture dura mater, and the CS suture associated dura mater all underlie a “sheet” of bone in vivo: the parietal bones or the fused coronal suture. Therefore, the ALP data may be

explained in that the only harvest site associated with a large pool of osteoprogenitor cells would have been the WT patent suture.

Regardless of the regional differences, it is important to notice that BMP stimulation had no significant effect on ALP expression in any of the dura mater-derived cells tested, evidenced by a lack of a significant dose main effect. This suggested that the dura mater-derived cells from CS rabbits were not more susceptible to osteogenic stimulation. Together with the proliferation data, the ALP data suggest that CS dura mater cells were not particularly different compared to WT cells.

The other means by which the dura mater may control suture patency is by direct control of the differentiation of bone cells. In fact, there is evidence that dura mater cells associated with fusing sutures can increase the differentiation of bone cells compared to dura mater cells associated with patent sutures¹⁸. There is also evidence that the dura mater in the CS rabbit model may be expressing different growth factors compared to WT dura mater^{31,36}. Therefore, we used co-culture to test whether the CS dura mater-derived cells were expressing soluble factors that increased osteogenesis in bone-derived cells. Our data suggest that CS dura mater cells do not express factors that induce osteogenesis in all rabbit bone-derived cells. Rather, these cells induced increased ALP activity *only in CS bone-derived cells*.

Co-culture with WT dura mater-derived cells led to a decrease in ALP activity in both WT and CS bone cells compared to bone cells not in co-culture, suggesting that CS bone cells were not hyper-reactive to a normal signal expressed by dura mater-derived cells. These and previously reported data suggest that CS rabbit bone-derived cells are similar to WT bone-derived cells in many respects³³. Co-culture with dura mater-derived cells seems to decrease ALP activity, in general. CS dura mater-derived cells do not seem to over-express osteogenic factors compared to WT dura mater cells because they induced a decrease in ALP activity in WT bone cells. The single culture condition that was different from the rest was the CS dura mater-derived cells co-cultured with CS bone cells, suggesting that cells from *both tissues* are necessary to display an aberrant phenotype. Such an interaction between tissues could derive from either a soluble protein-based or a non protein-based signal, that is expressed by one or both of the tissues. As stated previously, the results from the control groups argue against this interpretation in that no single tissue evoked a particular response³³.

With this evidence of a biological effect stemming from the CS dura mater in vitro, we tested whether disruption of signaling within the suturectomy site would influence postoperative resynostosis. Our transplant study showed that a synostotic suturectomy site would undergo resynostosis if left untreated or if a patent suture was placed within it. However, placement of a PTFE (gore-tex) membrane to cover the CS dura mater inhibited resynostosis of the suturectomy site and was shown to maintain the patency of transplanted patent sutures. Taken together with our previous transplant data,³¹ it seems likely that transplanted dura mater acts as a barrier to either soluble or physical signals from the CS dura mater, thereby inhibiting resynostosis.

Future studies should investigate the utility of barriers that may be useful in disrupting the molecular communication between these tissues. It is likely that physical or chemical barriers could be used to disrupt the communication between the dura mater and the overlying tissue. We previously demonstrated that transplanted normal dura mater into a craniosynostotic suturectomy site inhibited postoperative resynostosis, though it is unclear whether the dura acted as a physical or a molecular barrier³¹. We have recently demonstrated that molecular blockade of TGF- β 2 signaling using a neutralizing antibody³²

and blocking BMP signaling using noggin administration³⁷ were capable of inhibiting postoperative resynostosis, lending support to the concept of a molecular blockade being useful to improve management of craniosynostosis. We intend to identify the communicating signals between craniosynostotic dura mater and bone in order to develop effective therapies to inhibit postoperative resynostosis.

The results of this study support the hypothesis that CS dura mater-derived cells are more proliferative than WT cells, but do not support the hypotheses that CS dura mater cells are more ALP positive or respond more to osteogenic stimulation. Studies are underway to identify proteins within the medium shared by CS dura mater- and bone-derived cells that may be responsible for the increased ALP activity in culture. In addition, gene and protein expression analyses are being conducted to compare cells cultured in isolation and in co-culture.

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Figure 1.

Schematic diagram of the surgical procedure used. Note that the suturectomy site on the synostosed side was treated with: 1) nothing (suturectomy control), 2) patent suture from the opposite side of the same animal (suture only), 3) PTFE membrane (barrier only), or with 4) a patent suture and a PTFE membrane (suture and membrane).

Cells from CS dura proliferate faster than cells from WT dura

Figure 2.

Graph showing the mean absorbance ($\pm$ SEM) of WT or CS dura mater-derived cells cultured in one of three different media. PM = proliferation medium (control); BMP = proliferation medium + BMP4; OM = osteogenic medium. Note that CS dura mater-derived cells had more cells in each of the media compared to WT.

WT dura mater cells have higher ALP compared to CS dura mater cells

Figure 3.

Graph (above) and histological images (below; ALP stain) showing ALP activity of WT and CS dura mater cells derived from suture-associated (Suture) and non-suture-associated (Non-suture) regions. Notice that, overall, dura mater-derived cells from regions under bone (WT and CS non-suture, CS suture [fused]) were different from dura mater-derived cells from regions under a patent suture (WT suture).

Figure 4.

Histological images (left) and graph (right) of ALP activity assessed in bone cells cultured alone (control) or co-cultured with WT dura mater- or CS dura mater-derived cells. Note that co-culture with CS dura mater-derived cells increased the ALP activity of CS bone-derived cells only. All other co-culture conditions showed lower ALP activity compared to controls.

Figure 5.

Histological analysis of calvarial bone suturectomy site healing. Note that transplantation of a patent suture leads to fusion of the transplanted suture (Suture Only), implantation of a PTFE membrane inhibited bone growth across the suturectomy site (Barrier Only), and implantation of the PTFE membrane underlying the transplanted patent suture was capable of maintaining suture patency (Suture and Barrier).