BASIC SCIENCE: OBSTETRICS

Amniotic fluid levels of glial fibrillary acidic protein in fetal rats with retinoic acid induced myelomeningocele: a potential marker for spinal cord injury

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OBJECTIVE: The objective of this study was to determine whether amniotic fluid levels of glial acidic fibrillary protein (GFAP) would reflect myelomeningocele-related neurodegeneration in the rat model of retinoic acid–induced myelomeningocele, which is a model that is very similar to human myelomeningocele and develops the entire spectrum of disease severity including features of the Chiari II malformation.

STUDY DESIGN: Time-dated (embryonic day 10) pregnant Sprague-Dawley rats were gavage fed 60 mg/kg/bodyweight retinoic acid that had been dissolved in olive oil or olive oil alone. Myelomeningocele, retinoic acid–exposed no myelomeningocele, and control fetuses were harvested at specific time points throughout gestation. A standard set of pinching tests was performed to interrogate the sensorimotor reflex arc of hindpaws and tails. Amniotic fluid–GFAP levels were analyzed by standard enzyme-linked immunosorbent assay techniques.

RESULTS: Amniotic fluid–GFAP levels were similar between groups at embryonic days 14, 16, and 18, respectively. Compared with control fetuses, amniotic fluid GFAP levels were significantly increased in myelomeningocele fetuses at embryonic days 20 and 22 ($P < .001$). Defect size ($P < .001$), presence of clubfoot deformity ($P = .0004$), and absence of sensorimotor function ($P < .01$) at embryonic day 22 correlated with amniotic fluid–GFAP levels.

CONCLUSION: Amniotic fluid–GFAP levels appear to correlate with spinal cord injury as gestation proceeds in fetal rats with myelomeningocele.

Key words: amniotic fluid, fetal surgery, glial fibrillary acidic protein, myelomeningocele, neurologic function, retinoic acid

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Myelomeningocele is a devastating congenital malformation with complex physical and neurodevelopmental sequelae for which there is no cure. Myelomeningocele often results in life-long impairment that includes loss of sensorimotor function of the lower extremities, skeletal deformity, bladder and bowel incontinence, ventriculomegaly, and the Chiari II malformation.[1] Although the cause of myelomeningocele remains poorly understood, primary failure of either neural tube or mesenchymal closure at the caudal neuropore in the embryonic period results in exposure of the vulnerable neural elements to the intrauterine environment (first hit).[2] Without protective tissue coverage, secondary destruction of the exposed spinal cord by chemical and mechanical injury may occur throughout the remainder of gestation (second hit).[3,4] Theoretically, in utero coverage for the protection of the spinal cord might prevent the secondary component of the acquired damage, but not the primary injury. Preliminary, nonrandomized, and noncontrolled studies suggest that fetal myelomeningocele closure may improve neurologic function and reduce morbidity from hydrocephalus and the Chiari II malformation by reversal of the hindbrain herniation component in a subset of patients.[5-13] However, many children after fetal surgery have a residual deficit. Thus, the controversy in the context of prenatal therapy arises over patient selection, how much each of the 2 hits contributes to the observed neurologic deficits, and, from a practical standpoint, when during fetal development the secondary damage occurs.

Glial fibrillary acidic protein (GFAP) is a monomeric intermediate filament protein of the astroglial skeleton that is found in the white and gray brain and spinal cord matter and is strongly upregulated in astrogliosis. Recent evidence indicates that GFAP might be a useful marker for various types of brain and spinal cord damage that range from primary central nervous system malignancies and stroke to neurodegenerative disorders and traumatic spinal cord injury.[14-17] Additionally, several experimental studies suggest that the ongoing destruction of the vulnerable exposed spinal cord in myelomeningocele can be assessed by immunohistochemical examination of astrogliosis and astocyte activation that are associated with neu-

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rologic injury with anti-GFAP antibody.[18-20] Furthermore, it has been demonstrated that GFAP concentration is elevated within the amniotic fluid in the presence of myelomeningocele and other open neural tube defects.[21-24] We recently have developed a novel short-gestational animal model of isolated myelomeningocele in fetal rats by the maternal administration of retinoic acid (RA) and subsequently have demonstrated that exposure to RA at the time of posterior neuropore closure (embryonic day 10 [E10] in rats) leads to a pathologic condition that has striking morphologic and clinical similarity to human myelomeningocele.[25-27] As previously reported,[25-27] fetal rats that are exposed to RA experience myelomeningocele lesions that usually are confined to the lumbosacral area that is covered by a cystic sac with abnormal spinal cord tissue on the dorsal aspect and, histologically demonstrate loss of neural tissue, disruption of neural bundles, and areas of cord necrosis in the exposed segments, although the spinal cord and its coverings proximal to the defect remain normal. Clinically, fetal rats with myelomeningocele have clubfoot deformity, abnormal bladder and bowel function, and features of the Chiari II malformation. In contrast with surgical models, which only reproduce the acquired component of the spinal cord injury, prenatal administration of RA induces a primary defect during neural tube formation. Therefore, the RA-induced myelomeningocele model enables investigation of the evolution of abnormalities in the development of myelomeningocele from the point of defective neurulation forward. Because our ability to predict the severity of spinal cord injury in myelomeningocele currently is limited, we hypothesized that amniotic fluid (AF) levels of GFAP may reflect myelomeningocele-related neurodegeneration and abnormal neurofunction in fetal rats with RA-induced myelomeningocele.

Materials and Methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia and followed guidelines set forth in the *National Institutes of Health Guide for care and use of laboratory Animals. Animal preparation and retinoic acid exposure.*

The procedures for creating myelomeningocele defects have been described elsewhere.[25] Briefly, time-dated primigravid Sprague-Dawley rats (Charles River Laboratories, Inc, Wilmington, MA) were used for this study. After a brief exposure to isoflurane (Abbott Laboratories, North Chicago, IL), dams were gavage fed 50 mg/kg bodyweight of *all-trans* RA (Sigma-Aldrich Chemical, St. Louis, MO) that had been dissolved in 2 mL olive oil at E10. This dosage has been shown previously to induce isolated myelomeningocele-like defects in >60% of the offspring.[25-27] Control animals were fed 2 mL of olive oil.

Collection of fetuses and in vivo neurologic evaluation

Neurologic assessment of the sensorimotor function was performed as described previously by Stiefel et al.[28] Briefly, pregnant rats were anesthetized deeply by isoflurane and killed by cervical dislocation. Olive oil–exposed fetuses and fetuses with isolated myelomeningocele or no defects after RA exposure were used for analyses. Fetuses were delivered partially by cesarean section and maintained on placental support ex utero under a dissecting microscope. Neurofunctional testing was limited to the first 5 minutes after the death of the dam to ensure the vital reactions of the examined fetuses. Thus, only a maximum of three randomly selected fetuses per dam could be analyzed. Microforceps were used to perform the standardized pinching test of both forepaws, both hindpaws, and tail in all animals to elicit a classic pain reaction. Stimuli were repeated 3 times. Results were considered positive only if an immediate (within 1 second) and identical pain withdraw response was obtained on both upper and lower limbs and the tail in 3 consecutive test series. Because of physiologic immaturity of the nervous system for all animals at earlier stages,[29,30] neurofunctional investigation was possible only in fetuses at E18, E20, and at term (E22). After completion of the neurologic assessment fetuses were blotted dry, and bodyweight was recorded. In fetuses with myelomeningocele, the lesion was photographed; the craniocaudal and transverse diameters of the defect were measured, and the presence/absence of club foot deformity and a curly tail were recorded.

Collection of AF

In addition to the AF that was collected from fetuses that underwent neurofunctional assessment, AF from E14 and E16 fetuses was also collected for analysis of AF-GFAP concentration. Approximately 50 μL of AF was collected into a 1.5-mL Eppendorf tube (Eppendorf AG, Hamburg, Germany) per fetus. Samples were immediately snap-frozen in liquid nitrogen then stored at −70°C until further analysis.

Measurement of GFAP AF levels

AF-GFAP concentrations were analyzed with enzyme-linked immunosorbent assays (ScheBo Biotech AG, Giessen, Germany) according to the manufacturer's instructions. All samples were analyzed in duplicate. When high variation in duplicate results was observed for a sample, it was reassayed in triplicate.

General morphologic condition and immunohistochemistry

After fixation in 10% neutral buffered formalin (Sigma-Aldrich Chemical Company) for 5-10 days (depending on gestational age), fetuses were dehydrated in alcohol, paraffin embedded, and sectioned (4 μm). For general morphologic condition, sections were deparaffinized, rehydrated to distilled water, and stained with hematoxylin and eosin, according to standard protocols. Immunohistochemical techniques for immunofluorescence microscopy were performed as described previously.[26,27] Briefly, sections assigned for immunohistochemistry were deparaffinized, rehydrated, immersed in epitope unmasking solution (Vector Laboratories, Burlingame, CA), heated in a microwave, rinsed in distilled water, and blocked with peroxidase-blocking reagent (DakoCytomation, Glostrup, Denmark) for 30 minutes at room temperature before incubation overnight (4°C) with primary antibody that had been diluted in ready-to-use an-