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The effect of hemorrhage on the development of the postnatal mouse cerebellum

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ABSTRACT

Recent studies have shown that hemorrhagic injury in the preterm cerebellum leads to long-term neurological sequelae, such as motor, affective, and cognitive dysfunction. How cerebellar hemorrhage (CBH) affects the development and function of the cerebellum is largely unknown. Our study focuses on developing a mouse model of CBH to determine the anatomical, behavioral, and molecular phenotypes resulting from a hemorrhagic insult to the developing cerebellum. To induce CBH in the postnatal mouse cerebellum, we injected bacterial collagenase, which breaks down surrounding blood vessel walls, into the fourth ventricle at postnatal day two. We found a reduction in cerebellar size during postnatal growth, a decrease in granule cells, and persistent neurobehavioural abnormalities similar to abnormalities reported in preterm infants with CBH. We further investigated the molecular pathways that may be perturbed due to postnatal CBH and found a significant upregulation of genes in the inflammatory and sonic hedgehog pathway. These results point to an activation of endogenous mechanisms of injury and neuroprotection in response to postnatal CBH. Our study provides a preclinical model of CBH that may be used to understand the pathophysiology of preterm CBH and for potential development of preventive therapies and treatments.

Introduction

The incidence of preterm birth is approximately 11% worldwide (Blencowe et al., 2012). The survivors of premature birth are at an increased risk for various neurodevelopmental disabilities, such as motor deficits (i.e., cerebral palsy), sensory deficits (i.e., visual and auditory impairments), and cognitive, behavioral and socialization deficits (Wood et al., 2005). With the recent improvement in neuroimaging technology and more frequent use of magnetic resonance imaging (MRI), cerebellar abnormalities have become increasingly recognized as one of the major risk factors for these neurodevelopmental problems (Limperopoulos et al., 2007; Volpe, 2009). In particular, cerebellar hemorrhage (CBH) has been reported to be significantly associated with long-term neurological and behavioral sequelae. Recent studies have reported the incidence of CBH in preterm infant is 7–8% (Dyet et al., 2006; Tam et al., 2011), and among the infants who weigh less than 750 g at birth, the incidence rate was found to be as high as 17% (Limperopoulos et al., 2005).

The hemorrhagic injury to the preterm cerebellum most frequently occurs from the rupture of fragile immature blood vessels that surround the cerebellar germinal matrices, the external granule layer (EGL) and ventricular zone (VZ) (Volpe, 2008). During 24 to 40 weeks gestation, granule cells proliferate in the EGL, whereas the interneurons are generated in the VZ. The disruption of this rapid developmental program due to CBH may result in long-term structural and functional alterations in the cerebellum (Volpe, 2009). Indeed, preterm CBH has been shown to cause significant cerebellar volume reduction (Limperopoulos et al., 2010; Messerschmidt et al., 2005), and long-term neurological and behavioral disturbances, such as motor, cognitive, affective, and social function deficits (Limperopoulos et al., 2005, 2007).

Despite growing recognition of preterm CBH and its neurodevelopmental outcomes, it is largely unknown how CBH affects the development and function of the cerebellum. To date, detailed examination of the histological and molecular phenotypes that result from CBH has not been possible due to limitations of clinical studies and lack of animal models. Thus, it is clinically important to develop and characterize an animal model of preterm CBH to understand the effects of CBH on the developing postnatal cerebellum.

In the present study, we developed an animal model of preterm CBH in neonatal mouse pups. We used pups at postnatal day two (P2), which is developmentally comparable to 26–30 weeks gestation in human (Biran et al., 2012). Using this animal model, we examined anatomical features of the cerebellum, behavioral phenotypes, and expression levels of genes that are relevant to brain injury and neuroprotection after hemorrhagic insult. We hypothesized that postnatal CBH leads to abnormalities in cerebellar development, deficits in cerebellar-specific

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behaviors, and the activation of molecular pathways for brain injury and neuroprotection.

Materials and methods

Animals

This study was conducted in accordance with the guidelines defined by the Canadian Council of Animal Care and was approved by the University of British Columbia Animal Care Committee. In this study, we used ICR (CD1) mice, which first originated from a group of Swiss mice consisting of two males and seven female albino mice derived from a non-inbred stock in the laboratory of Dr. de Coulon, Centre Anticancereux Romand, Lausanne, Switzerland. These animals were imported to the United States by Dr. Clara Lynch of the Rockefeller Institute in 1926. The Hauschka (Ha/ICR) stock was initiated in 1948 at the Institute for Cancer Research (ICR) in Philadelphia from "Swiss" mice of Rockefeller origin. Adult female mice (P60–P150) were individually mated with a male stud. Pregnant females were group housed until E17, upon which they were singly housed for the duration of pregnancy and lactation. The day of birth was considered P0. Pups were kept with their mother until wean age, P21.

Determination of method of cerebellar hemorrhage induction and collagenase dose

CBH was induced in the pups at P2. Before inducing the hemorrhage, mice were anesthetized with isoflurane (4% isoflurane in oxygen for induction, 1–2% isoflurane in oxygen for maintenance, from a precision vaporizer). To determine the best method to induce cerebellar hemorrhage in postnatal pups, l μl of autologous blood, 1 μl of bacterial collagenase (Sigma-Aldrich), or 1 μl of sterile saline was injected into the cerebral aqueduct (using a 10 μl Hamilton syringe) of individual mouse pups. The cerebral aqueduct is continuous with the fourth ventricle (Fig. 1A). Injection of autologous blood did not induce pronounced hemorrhaging in the postnatal brain, nor contribute to any area differences in the postnatal cerebellum. Bacterial collagenase was dissolved in sterile saline to obtain a concentration of 2 U/μl. We then serially dilute the 2 U/μl stock to give concentrations of 1 U/μl, 0.5 U/μl, 0.1 U/μl, and 0.05 U/μl. We found that 1 U/μl, 0.5 U/μl, and 0.1 U/μl of collagenase induced 100% mortality in P2 pups when injected into the cerebral aqueduct, whereas 0.05 U/μl of collagenase did not induce hemorrhaging. We then determined whether 0.075 U/μl, 0.06 U/μl, and 0.057 U/μl of collagenase would be more appropriate concentrations of collagenase to inject into the cerebral aqueduct. We found that a concentration of 0.057 U/μl would provide an adequate number of surviving P2 pups within a litter, post-hemorrhage induction, and that appropriate hemorrhaging in the ventricular system of the postnatal brain was present after collagenase delivery. This dose of collagenase (0.057 U/μl) was used for all of the experiments reported in this study. After delivering this dose of collagenase, the syringe remained in place for 2 min to prevent back-leakage before being withdrawn. Controls were injected with 1 μl of saline, using the same injection methods. Animals were placed on a warmed heating pad until active forelimb and hindlimb movement were regained upon gently touching pups. Pups were then returned to their nest with the mother following recovery from

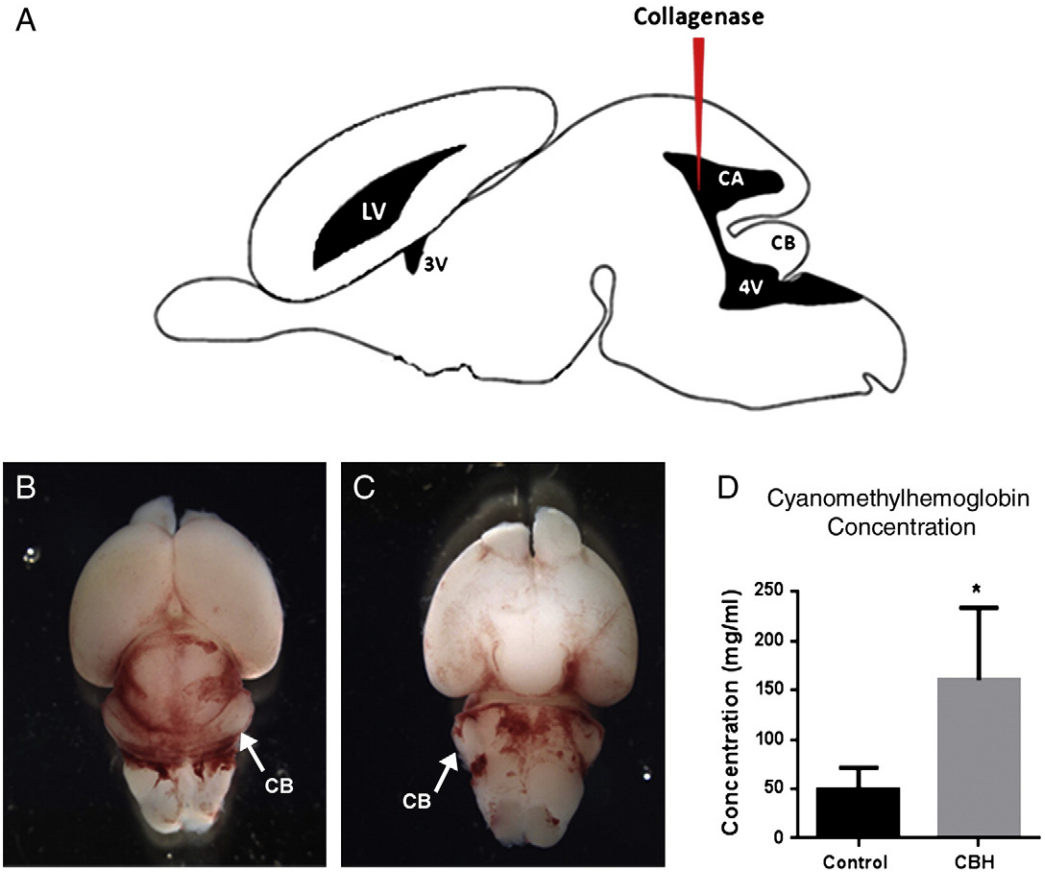

Fig. 1. The induction of cerebellar hemorrhage with collagenase injection. (A) Bacterial collagenase was injected into the cerebral aqueduct (CA), which subsequently would flow into the fourth ventricle (4 V). (B) Dorsal and (C) ventral brain shows blood accumulation on the surface of the cerebellum (CB) 30 min after the induction of the hemorrhage. (D) Spectrophotometric hemoglobin assay results show significantly higher concentration of cyanomethylhemoglobin in CBH brains. Mean ± SEM and *P < 0.05. $n = 6$ for control and $n = 7$ for CBH.