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Intraventricular hemorrhage induces deposition of proteoglycans in premature rabbits, but their *in vivo* degradation with chondroitinase does not restore myelination, ventricle size and neurological recovery

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ABSTRACT

Intraventricular hemorrhage (IVH) results in white matter injury and hydrocephalus in premature infants. Chondroitin sulfate proteoglycans (CSPGs)–neuorcan, brevican, versican, aggrecan and phosphacan–are unregulated in the extracellular matrix after brain injury, and their degradation enhances plasticity of the brain. Therefore, we hypothesized that CSPG levels were elevated in the forebrain of premature infants with IVH and that *in vivo* degradation of CSPGs would enhance maturation of oligodendrocyte, augment myelination, promote neurological recovery, and minimize hydrocephalus. We found that levels of neurocan, brevican, aggrecan, phosphacan, and versican were elevated, whereas NG2 expression was reduced in premature rabbit pups and human infants with IVH compared to controls. Intracerebroventricular chondroitinase ABC (ChABC) reduced the expression of neuorcan, brevican, versican and aggrecan, but not NG2. However, ChABC treatment did not enhance maturation of oligodendrocytes, myelination, or neurological recovery in the pups with IVH. Moreover, ChABC did not reduce gliosis or ventriculomegaly. Our results demonstrate that IVH induces distinct changes in the components of CSPGs, and that reversing these changes by *in vivo* ChABC treatment neither promotes clinical recovery, myelination, nor reduces ventriculomegaly in preterm rabbit pups.

Introduction

Intraventricular hemorrhage (IVH) remains a major cause of both morbidity and mortality in premature infants. IVH results in post-hemorrhagic hydrocephalus and reduced myelination of the white matter. The development of hydrocephalus is attributed to the obstruction of CSF flow by blood clots and impaired CSF absorption in the cerebral ventricles and subarachnoid space; and the hypomyelination is ascribed to degeneration and arrested maturation of oligodendroglial progenitors in the periventricular white matter (Dummula et al., 2011). Chondroitin sulfate proteoglycans (CSPGs) are major component of extracellular matrix (ECM) in the brain and are upregulated after brain injury around the site of lesion (Morgenstern et al., 2002). Elevated CSPGs in the ECM reduce migration and formation of process-outgrowth of oligodendrocyte (OL) progenitors, and impede movement of extracellular fluid in the brain (Siebert and Osterhout, 2011; Sykova et al., 2001). Therefore, we specifically asked whether CSPG levels were elevated in the forebrain of premature infants with IVH, and determined whether degradation of CSPG would restore myelination and minimize hydrocephalus in these infants.

CSPG consists of a core protein that is covalently linked to glycosaminoglycan (GAG) side chains. The CSPGs include lecticans, phosphacan and NG2; and the families of lecticans comprise aggrecan, versican, neurocan and brevican. CSPGs are elevated in animal models of cerebral hypoxia–ischemia, surgically injured cerebral cortex and spinal cord, and inflammatory brain disease (Li et al., 2008; Matsui et al., 2005). CSPG restricts plasticity of the brain after injury; and degradation of CSPGs by chondroitinase ABC (ChABC) treatment enhances plasticity of the brain structures. Indeed, ChABC promotes sprouting of axons in the spinal cord and nigrostriatal tract injury, stabilizes synaptic contacts, and enhances outgrowth of Purkinje cell neurites in the cerebellum (Bradbury et al., 2002; Hunanyan et al., 2010; Moon et al., 2001). ChABC treatment removes the GAG chain and thus, allows the CSPG core protein to be cleared by plasmin proteolytic system and other endogenous proteases (Bukhari et al., 2011; Tsirka et al., 1997).

Elevated CSPGs affect proliferation of neural cells and maturation of OL (Gu et al., 2009). In culture experiments, elevated CSPGs inhibit

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migration, formation of process outgrowth, and differentiation of OL progenitor cells, thereby contributing to myelination failure (Siebert and Osterhout, 2011; Silver and Miller, 2004). Maturation of OL progenitors into mature OL requires both extension of processes to ensheathe axons and expression of myelin proteins. CSPGs, including neurocan and phosphacan, strongly inhibit OL process outgrowth (Siebert and Osterhout, 2011). In addition, CSPG forms a significant barrier to the migration of OL progenitors. Consistent to these findings, ChABC treatment degrades CSPGs and promotes both migration as well as differentiation of OL in culture experiments and animal models of spinal cord injuries (Siebert et al., 2011).

CSPGs are elevated in cerebrospinal fluid of preterm infants with post-hemorrhagic hydrocephalus, suggesting that CSPGs might be released into the CSF by the periventricular neural cells (Chow et al., 2005). Importantly, accumulation of CSPGs in the ECM might reduce the flow of extracellular fluid and contribute to hydrocephalus (Del Bigio and Enno, 2008; Massicotte et al., 2000; Shoesmith et al., 2000). Despite this, CSPGs have neither been evaluated in the cerebral cortex of brain injury models of hypomyelination nor in a paradigm of post-hemorrhagic hydrocephalus. Therefore, we hypothesized that CSPGs were elevated in the forebrain of premature newborns with IVH, and that degradation of CSPGs by ChABC treatment might enhance maturation of OL, myelination, and clinical recovery in premature infants with IVH. We also postulated that ChABC treatment would minimize hydrocephalus. To test our hypotheses, we used our animal model, in which rabbit pups (E29, term = 32d) with glycerol-induced IVH exhibit arrested OL maturation, hypomyelination and hydrocephalus at 2-week age (Chua et al., 2009). We found that CSPGs, including brevican, neurocan, aggrecan, phosphacan, and versican were elevated, whereas NG2 levels were reduced in pups with IVH compared to controls. More importantly, degradation of CSPGs by *in vivo* ChABC treatment neither promoted myelination, clinical recovery, nor reduced gliosis and ventriculomegaly.

Material and methods

Animal experiments

The Institutional Animal Care and Use Committee of New York Medical College approved all experimental protocols. Timed-pregnant New Zealand rabbits were obtained from Charles River Laboratories, Inc. (Wilmington, Mass., USA). Cesarean-section was performed to deliver the pups prematurely at E29 (full-term = 32 days). We kept them in an infant incubator that was pre-warmed to a temperature of 35 °C. The pups were gavage-fed 2–4 ml (100 ml/kg) of puppy formula (Esbilac, PetAg, IL) twice a day and then feeds were advanced to 125, 150, 200, 250 and 280 ml/kg at postnatal days 3, 5, 7, 10 and 14 respectively. The rabbit pups of either sex were administered 50% glycerol (6.5 g/kg) intraperitoneally at 2–3 h of age to induce IVH. Head ultrasound was performed at 6 h of age to assess the presence and severity of IVH using an Acuson Sequoia C256 (Siemens) ultrasound machine. Severity of IVH was diagnosed by measuring ventricle volume (length, breadth & depth in coronal & sagittal views) on head ultrasound at 6 h age. Pups were stratified based on ventricular volume into moderate (30–150 mm^3) and severe (151–250 mm^3) IVH. Ventricular volume <30 mm^3 indicates microscopic or no IVH. The pups with moderate and severe IVH were assigned to treatment and control group in such a manner that the severity of IVH was balanced between the comparison groups. To determine the effect of IVH on the expression of CSPGs, we compared pups with IVH and glycerol-treated control pups without IVH.

In vivo ChABC treatment

The rabbit pups with IVH were sequentially treated with either 20 μl ChABC (50 U/ml, catalog # C3667, Sigma, St Louis, USA) or vehicle at days 2, 4 and 6. ChABC or vehicle (saline) was administered intracerebroventricularly. Briefly, the pups were mounted on rabbit pup restrainer after anesthetizing them with ketamine and xylazine. A needle of 27 gauge with 50 μl Hamilton syringe was mounted on a micromanipulator to inject the ChABC into the ventricle. We used the following coordinates from bregma: 1 mm anterior, 4 mm lateral, and 3 mm deep. The dose of ChABC was calculated based on its previous use in rats to treat spinal cord and cerebral injury (Bradbury et al., 2002; Bruckner et al., 1998; Harris et al., 2009) and our initial experiments showing reduction in CSPG core-protein by 95% on ChABC treatment (Supplementary Fig. 1). The severity of IVH, measured by ultrasound, was similar between the comparison groups—vehicle treated pups with IVH and ChABC-treated pups with IVH (ventricular volume 0.170 ± 0.02 *vs.* 0.174 ± 0.03 cm^3).

Human subjects

The Institutional Review Board of New York Medical College, Valhalla, NY approved the use of autopsy materials from premature infants for this study. The study materials included forebrain tissue samples taken from the premature infants with and without IVH of 23–27 weeks of gestational age and 7–14 days of postnatal age (Table 1). Samples were obtained less than 24 h postmortem. We excluded premature infants with meningitis, hypoxic–ischemic encephalopathy, culture proven sepsis, major congenital anomalies and chromosomal defects. We included 4–5 infants in each group—IVH and no IVH. The wall of the cerebral hemisphere in premature infants consists of ventricular zone (VZ), subventricular zone (SVZ), intermediate zone, cortical plate, and marginal zone as described by the Boulder Committee (Bystron et al., 2008). In the present study, we described intermediate-zone embryonic white matter synonymously with white matter and cortex for the cortical plate.

Rabbit tissue collection and processing

We processed the tissues as we previously described (Ballabh et al., 2007). The brain slices were immersion-fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; 0.1 M, pH 7.4) overnight and

Table 1
Characteristics of human infants with and without IVH.

Postconceptional age (weeks)	Sex	Birth weight (kg)	IVH/no IVH	Cause of death
26	Male	0.810	Grade 3 IVH	Clinical sepsis
23	Male	0.57	Grade 2 IVH	Clinical sepsis
23	Female	0.58	Grade 3 IVH	Respiratory failure
24	Male	0.64	Grade 4 IVH	Pulmonary hemorrhage
23	Male	0.45	No IVH	RDS, respiratory failure
24	Male	0.61	No IVH	Clinical sepsis
25	Male	0.73	No IVH	Metabolic acidosis, resp. failure
27	Female	0.56	No IVH	Respiratory failure