

AD_____

Award Number: W81XWH-10-1-0967

TITLE: Hyaluronan oligosaccharides for the promotion of remyelination

PRINCIPAL INVESTIGATOR: Larry S. Sherman & Paul H. Weigel

CONTRACTING ORGANIZATION:
Oregon Health & Science University
Portland, OR 97201-4762

REPORT DATE: October 2012

TYPE OF REPORT: FINAL (Revised)

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2012		2. REPORT TYPE FINAL (revised)		3. DATES COVERED 30September2010-29September2012	
4. TITLE AND SUBTITLE Hyaluronan oligosaccharides for the promotion of remyelination				5a. CONTRACT NUMBER W81XWH-10-1-0967	
				5b. GRANT NUMBER W81XWH-10-1-0967	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Larry S. Sherman and Paul H. Weigel E-Mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Oregon Health & Science University Portland, OR 97201-4762				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The overall goal of this project was to determine if digestion products of the glycosaminoglycan hyaluronan (HA) generated by hyaluronidases expressed by oligodendrocyte progenitor cells (OPCs) block OPC differentiation and remyelination; and if particular formulations of HA oligosaccharides could block the activities of these digestion products. During the course of this study, we determined that a specific hyaluronidase, PH20, is induced in OPCs and astrocytes in demyelinating lesions; that PH20 generates a unique profile of HA digestion products; that these products are sufficient to inhibit OPC maturation and remyelination; and that blocking PH20 activity can promote remyelination. These findings are now in press in Annals of Neurology and suggest a new drug target for the promotion of remyelination in multiple sclerosis and related demyelinating diseases.					
15. SUBJECT TERMS multiple sclerosis, hyaluronan, hyaluronidase, remyelination					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	22	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	3
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusions.....	8
References.....	8
Appendix.....	9

INTRODUCTION

The destruction of myelin in the central nervous system (CNS) is a hallmark of multiple sclerosis (MS). Remyelination failure in demyelinated MS lesions contributes to axonal loss, which is the major pathological correlate of progressive functional decline in MS patients. **Thus, developing strategies that promote remyelination is an important goal in the treatment of MS.**

The Sherman lab previously discovered that a high molecular weight form of the glycosaminoglycan hyaluronan (HA) accumulates in demyelinated lesions from patients with MS and in mice with experimental autoimmune encephalomyelitis (EAE). They further found that this form of HA prevents remyelination in chemically demyelinated lesions and that it inhibits oligodendrocyte progenitor cell (OPC) maturation. These data were consistent with the hypothesis that high molecular weight HA itself inhibited OPC maturation, preventing oligodendrocytes from remyelinating demyelinated axons.

We tested this hypothesis by pre-treating high molecular weight HA with different preparations of hyaluronidases, then determining if the break-down products of these hyaluronidases could no longer inhibit remyelination. Surprisingly, we found that preparations of high molecular weight HA treated with bovine testicular hyaluronidase potently inhibited remyelination *in vivo* and blocked OPC maturation *in vitro*. This effect was not observed following treatment of HA with *Streptomyces* hyaluronidase. We also found that OPCs that overexpressed particular hyaluronidases found in preparations of bovine testicular hyaluronidase failed to mature into myelinating oligodendrocytes, that OPCs express these hyaluronidases, and that OPCs can degrade the HA they encounter in demyelinated lesions.

Collectively, these preliminary data supported the hypothesis that the digestion products of HA generated by particular hyaluronidases are the signals that contribute to remyelination failure by inhibiting OPC maturation. We further hypothesize that interfering with interactions between these breakdown products and receptors on OPCs will promote OPC maturation and remyelination.

During the course of this project, we have developed protocols to assay which specific HA digestion products are generated by OPCs and mature oligodendrocytes (OLs) *in vitro*, and in spinal cord lesions from mice with EAE. We determined that a specific hyaluronidase, called PH20, is expressed by OPCs and astrocytes in demyelinated MS and EAE lesions and that digestion products of PH20 block remyelination. We have utilized the assays we developed in the first year of this project to assess the sizes and quantities of HA digestion products generated by PH20. We have generated endotoxin-low, highly enriched preparations of HA and determined the precise size ranges of HA that block OPC maturation. Although we did not find that HA oligosaccharides were capable of blocking the effects of PH20-generated HA digestion products on OPC maturation, we did find that a pharmacological inhibitor of hyaluronidase activity could promote OPC maturation and functional remyelination. **All together, these data indicate that targeting PH20 is a potentially efficacious way to promote remyelination in MS and other demyelinating diseases.**

BODY

Task 1: Examine the patterns of hyaluronan (HA) degradation products by oligodendrocyte (OL) progenitors in vitro and within spinal cord lesions of mice with experimental autoimmune encephalomyelitis (EAE)

Our analyses of HA digestion products in OPC cultures demonstrated that HA content in mature oligodendrocyte cultures was higher than in immature OPC and pre-oligodendrocyte cultures (Table 1). We subsequently determined that OPCs have distinct hyaluronidase activity that was attributed to the PH20 hyaluronidase (Preston et al., 2013). Analysis of the molecular weight species of HA digestion products generated by PH20 (using bovine testicular hyaluronidase as a PH20 source – BTH) revealed that PH20 digestions products are distinct from *Streptomyces* (StrepH) hyaluronidase (Figure 1; Preston et al., 2013).

Table 1. HA content of OL and OPC culture medium

Sample	Mean $\pm$ s.d. (ng/ml)
Pre Ols	219 $\pm$ 40
OPCs	100 $\pm$ 12
OLs	826 $\pm$ 82

Numerous attempts to fully analyze the distribution of HA digestion products in white matter tissues failed to produce definitive results. However, we were able to determine that spinal cord tissues isolated from mice with EAE demonstrated increased hyaluronidase activity compared to control tissues. Lumbar spinal cord sections from mice with active EAE (14 days post-inoculation) and the same lumbar sections from control animals were lysed and incubated with high molecular weight (> 1 MDa) HA. There was 20-30% more HA digested by the EAE spinal cord lysates than by the control lysates as assessed by agarose gel electrophoresis as previously described (Lee and Cowman, 1994) followed by quantification of digitized images using ImageJ (Figure 2).

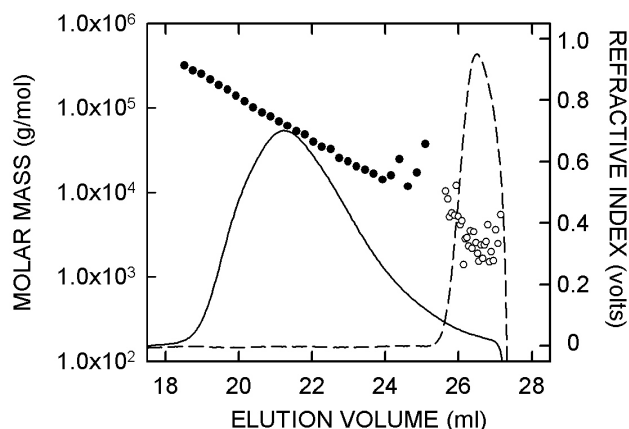


Figure 1. Product size distributions of BTH and StrepH digests are different. HA samples were treated with BTH (solid line and black circles) or StrepH (dashed line and white circles) and analyzed by SEC-MALLS to determine the amount (refractive index, solid and dashed lines) and the molar masses based on light scattering (black and white circles) of HA products present. The weight-average masses of the BTH and StrepH samples were 63.9 ± 0.5 kDa and 2.4 ± 0.4 kDa, respectively. Overlap of the two digests was $\leq 2\%$ (e.g. 98% of the BTH products ranged from 5.5 to 227 kDa and 98% of the StrepH products were < 4.4 kDa).

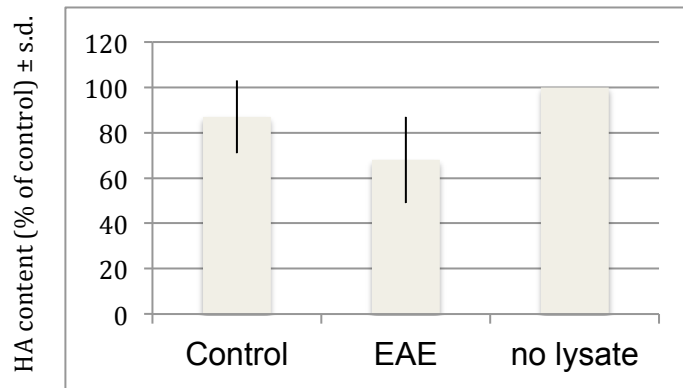


Figure 2. Hyaluronidase activity in control vs. EAE spinal cords. Lysates of lumbar spinal cords were mixed with high MW HA and incubated at 37°C for 1 hr. then analyzed for HA content using an ELISA-based assay. Control: spinal cord tissue from age-matched control mouse; EAE: tissue from mouse with EAE at 12 days post-inoculation; no lysate: HA incubated in buffer alone.

In summary, the studies performed in task 1 demonstrated that (a) OPCs digest HA through elevated expression of the PH20 hyaluronidase; (b) that PH20 generates specific sized digestion products of HA; and (c) that hyaluronidase activity is elevated in tissues from mice with demyelinating EAE lesions.

Task 2: Test if specific combinations of HA degradation products are sufficient to block remyelination and oligodendrocyte progenitor cell maturation in vivo.

We tested if the digestion products of HA generated by PH20, and not those generated by *Streptomyces* hyaluronidase, could block OPC maturation and remyelination. Only products generated by PH20 blocked OPC maturation in vitro and remyelination in lysolecithin lesions where HA was present (Preston et al., 2013). We next aimed to determine which HA digestion products could block OPC maturation. The Weigel lab generated endotoxin-free samples of different sizes of HA digestion products generated by incubation of high MW HA with PH20 (Table 2). Endotoxin tests were performed according to the manufacturer's instructions using the Charles River Lab (Wilmington, MA) Kinetic Turbidimetric Assay.

Table 2: Average size and size range for final HA pools

Pool	Mw (kDa)	1-99 % (kDa)	mg		Pool	Mw (kDa)	1-99% (kDa)	mg
A	126 ± 3	83 - 155	1.27		H	44 ± 1	31.1 - 53.2	3.54
B	102	71 - 121	1.18		I	36 ± 1	24.4 - 45.5	3.47
D	87 ± 5	67 - 95	1.41		J	28 ± 0.3	17.0 - 38.1	6.83
E	74 ± 2	60 - 82	1.92		K	20 ± 1	11.8 - 29.4	4.95
F	59	47 - 69	4.33		L	13 ± 1	~9 - 21	3.90
G	51 ± 1	38 - 60	3.74		M	10 ± 2	~7 - 14	1.37

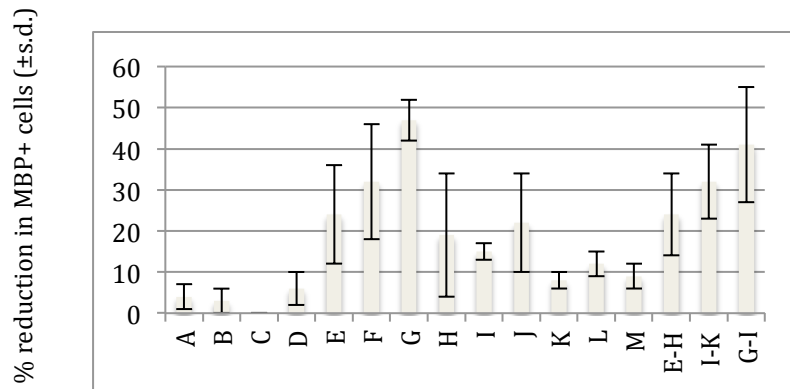


Figure 3. Effects of different sizes of HA digestion products on OPC maturation.

Fractions or mixtures of fractions from Table 2 were tested in OPC cultures and the % of cells expressing MBP compared to controls was determined.

Individual fractions of HA were tested for their effects on OPC maturation *in vitro*. We found that fractions E-J (Table 2) each had the greatest inhibitory activity while combinations of these fractions (e.g. E-H, I-K, G-I) all had similar degrees of inhibitory activity (Figure 3). Fractions K-M also weakly inhibited OPC maturation. These findings support a new model for a molecular mechanism underlying remyelination failure where HA accumulates coincident with reactive astrogliosis in demyelinating lesions; OPCs that express PH20 are recruited to lesions; PH20-generated HA digestion products signal to OPCs, preventing their maturation to myelin-forming oligodendrocytes, thus preventing remyelination.

Task 3: Test if specific formulations of HA oligosaccharides can promote oligodendrocyte progenitor cell maturation in vitro and in vivo

Using enriched fractions of PH20-digested HA isolated and prepared in the Weigel laboratory, we tested the effects of these fractions on OPC maturation *in vitro* and whether any of them could interfere with the effects of PH20 on OPC maturation. None of the fractions, either alone in combination, demonstrated the ability to reverse the effects of PH20 digestion on OPC maturation (data not shown).

Given these findings, we decided to test if a hyaluronidase inhibitor could promote OPC maturation. We used L-Ascorbic acid 6-hexadecanoate (also called VCPAL), which has potent hyaluronidase inhibitory activity (Botzki et al., 2004). Although not specific for PH20, we found that VCPAL could promote OPC maturation *in vitro* and remyelination in lysolecithin-induced lesions in the presence of HA (Figure 4). Furthermore, we found that VCPAL promoted increased conduction velocities in these lesions, indicating that blocking hyaluronidase activity can promote functional remyelination. These findings are described in Preston et al. (2013; attached).

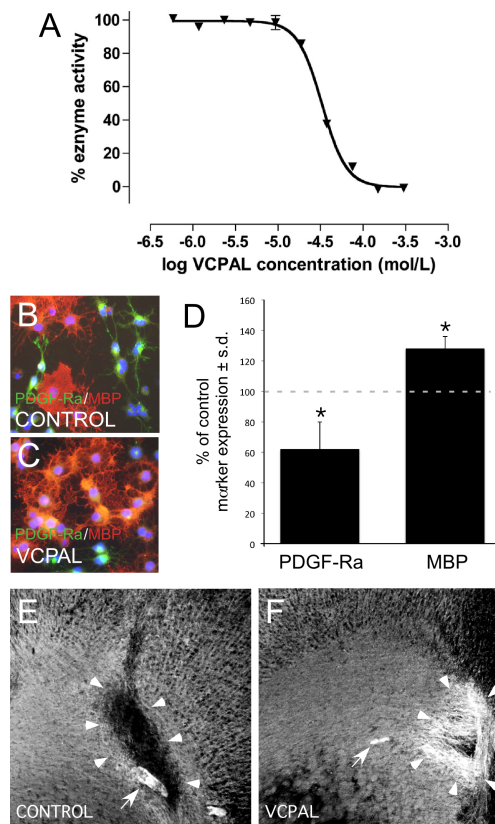


Figure 4. Inhibiting hyaluronidase activity promotes OL maturation *in vitro* and remyelination *in vivo*. (A) Inhibitory activity of increasing concentrations of the pan hyaluronidase inhibitor VCPAL (6-O-Palmitoyl-L-ascorbic acid) on BTH as determined using a turbidimetric assay. The final BTH concentration was 100 U/ml. Each point represents the mean±SEM of 4 replicate experiments. The VCPAL IC₅₀ was approximately 33 μM. (B) OPCs were grown in OL differentiation medium alone or (C) with 25μM VCPAL for 72-96 hrs and stained with MBP, PDGFRα and DAPI. Total MBP+ and PDGFRα+ cells are quantified in (D). Consistent with the hypothesis that hyaluronidases expressed by mOPCs generate HA fragments which inhibit OL maturation, blocking HYAL activity with VCPAL increased the total percentage of MBP+ cells compared to controls (69.31% v. 54.05% p= 0.00505) while decreasing the total percentage of PDGFRα+ cells (19.61% v. 30.98% in control, p=0.01719). (E, F) MBP immunoreactivity (white) of lysolecithin lesions treated with HMW HA and vehicle (E) or HMW HA with 25 μM VCPAL (F). Arrowheads indicate the borders of the lesions; arrows indicate non-specific staining of adjacent blood vessels.

KEY RESEARCH ACCOMPLISHMENTS

- Optimized pre-oligodendrocyte and OPC mouse culture system for isolating and characterizing HA
- Demonstrated that HA accumulates in oligodendrocyte cultures but not in OPC cultures, consistent with our findings that hyaluronidase activity is higher in immature vs. mature oligodendrocyte lineage cells
- Demonstrated the unique patterns of HA digestion products generated by bovine testicular hyaluronidase/PH20 vs. *Streptomyces* hyaluronidase
- Generated purified, low-endotoxin fragments of HA in fractions that include the range of HA digestion products generated by PH20
- Determined that lower-sized fragments of HA generated by PH20 block OPC maturation and remyelination
- Determined that HA oligosaccharides are unlikely to be effective for blocking the inhibitory activity of PH20-generated HA digestion products
- Determined that blocking hyaluronidase activity using VCPAL is sufficient to promote OPC maturation *in vitro* and remyelination in HA-rich demyelinated lesions *in vivo*

REPORTABLE OUTCOMES

The majority of the data reported here have been published in:

Preston M, Gong X, Su W, Matsumoto S, Banine F, Winkler C, Foster S, Xing R, Struve J, Dean J, Baggenstoss B, Weigel P, Montine T, Back S, Sherman LS. Digestion products of the PH20 hyaluronidase inhibit remyelination. *Ann. Neurol.* (attached)

CONCLUSIONS

This study has demonstrated, for the first time, that specific digestion products of HA generated by the PH20 hyaluronidase inhibit OPC maturation and prevent remyelination. Furthermore, this study demonstrated that although HA oligosaccharides may not be effective at reversing the effects of PH20 digestion products, blocking hyaluronidase activity can promote remyelination. These findings are highly significant in that they suggest that a specific inhibitor of PH20 may be an effective way to promote remyelination of spared axons within demyelinated lesions in MS patients and others with demyelinating disease.

Although we found that a broad-spectrum hyaluronidase inhibitor could promote remyelination, this agent (VCPAL) is unlikely to be useful as a drug to promote remyelination. Because it blocks all hyaluronidase activity, VCPAL could have serious side effects given the numerous roles played by some hyaluronidases throughout the body, including the joints and the cardiovascular system. Drugs that specifically target the hyaluronidase activity of PH20, however, would likely be far safer. PH20 is not normally expressed in the brain or at high levels elsewhere in the body other than in sperm. Thus, a tolerable side effect of chronic PH20 inhibition might be male infertility. We propose, therefore, that PH20-specific hyaluronidase inhibitors would be both safe and efficacious as a therapy or co-therapy to promote remyelination.

REFERENCES

Botzki A, Rigden DJ, Braun S, Nukui M, Salmen S, Hoechstetter J, Bernhardt G, Dove S, Jedrzejewski MJ, Buschauer A. L-Ascorbic acid 6-hexadecanoate, a potent hyaluronidase inhibitor. X-ray structure and molecular modeling of enzyme-inhibitor complexes. *J Biol Chem.* 2004 Oct 29;279(44):45990-7.

Lee HG, Cowman MK. An agarose gel electrophoretic method for analysis of hyaluronan molecular weight distribution. *Anal Biochem.* 1994 Jun;219(2):278-87.

Digestion Products of the PH20 Hyaluronidase Inhibit Remyelination

Marnie Preston, PhD,¹ Xi Gong, MD,² Weiping Su, MD, PhD,¹
 Steven G. Matsumoto, PhD,^{1,3} Fatima Banine, PhD,¹ Clayton Winkler, PhD,¹
 Scott Foster, BS,¹ Rubing Xing, BS,¹ Jaime Struve, BS,¹ Justin Dean, PhD,²
 Bruce Baggenstoss, BS,⁴ Paul H. Weigel, PhD,⁴ Thomas J. Montine, MD, PhD,⁵
 Stephen A. Back, MD, PhD,^{2,6} and Larry S. Sherman, PhD^{1,7}

Objective: Oligodendrocyte progenitor cells (OPCs) recruited to demyelinating lesions often fail to mature into oligodendrocytes (OLs) that remyelinate spared axons. The glycosaminoglycan hyaluronan (HA) accumulates in demyelinating lesions and has been implicated in the failure of OPC maturation and remyelination. We tested the hypothesis that OPCs in demyelinating lesions express a specific hyaluronidase, and that digestion products of this enzyme inhibit OPC maturation.

Methods: Mouse OPCs grown in vitro were analyzed for hyaluronidase expression and activity. Gain of function studies were used to define the hyaluronidases that blocked OPC maturation. Mouse and human demyelinating lesions were assessed for hyaluronidase expression. Digestion products from different hyaluronidases and a hyaluronidase inhibitor were tested for their effects on OPC maturation and functional remyelination in vivo.

Results: OPCs demonstrated hyaluronidase activity in vitro and expressed multiple hyaluronidases, including HYAL1, HYAL2, and PH20. HA digestion by PH20 but not other hyaluronidases inhibited OPC maturation into OLs. In contrast, inhibiting HA synthesis did not influence OPC maturation. PH20 expression was elevated in OPCs and reactive astrocytes in both rodent and human demyelinating lesions. HA digestion products generated by the PH20 hyaluronidase but not another hyaluronidase inhibited remyelination following lysolecithin-induced demyelination. Inhibition of hyaluronidase activity lead to increased OPC maturation and promoted increased conduction velocities through lesions.

Interpretation: We determined that PH20 is elevated in demyelinating lesions and that increased PH20 expression is sufficient to inhibit OPC maturation and remyelination. Pharmacological inhibition of PH20 may therefore be an effective way to promote remyelination in multiple sclerosis and related conditions.

ANN NEUROL 2013;73:266–280

Demyelination occurs following numerous insults to the central nervous system (CNS) and is the hallmark of multiple sclerosis (MS), causing conduction deficits that compromise motor, sensory, and cognitive functions. Some recovery of function is associated with the recruitment of oligodendrocyte progenitor cells (OPCs) to demyelinating lesions, generating oligodendrocytes (OLs) that remyelinate spared axons.¹ However, OPCs

often accumulate in chronically demyelinated lesions and fail to give rise to myelinating OLs.^{2–7} Strategies that promote OPC maturation within demyelinating lesions therefore have the potential to promote remyelination and functional recovery in affected individuals.

Multiple signals within the microenvironments of demyelinating lesions contribute to the failure of OPC maturation and remyelination.^{8,9} We previously found

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.23788

Received Dec 16, 2011, and in revised form Sep 25, 2012. Accepted for publication Oct 8, 2012.

Address correspondence to Dr Sherman, Division of Neuroscience, Oregon National Primate Research Center, 505 NW 185th Avenue, Beaverton, OR 97006. E-mail: shermanl@ohsu.edu

From the ¹Division of Neuroscience, Oregon National Primate Research Center, Beaverton, OR; ²Department of Pediatrics, Oregon Health and Science University, Portland, OR; ³Integrative Bioscience Department, School of Dentistry, Oregon Health and Science University, Portland, OR; ⁴Department of Biochemistry and Molecular Biology and the Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK; ⁵Department of Pathology, University of Washington, Seattle, WA; ⁶Department of Neurology and ⁷Department of Cell and Developmental Biology, Oregon Health and Science University, Portland, OR.

Additional supporting information can be found in the online version of this article.

that high molecular weight (HMW) forms of the glycosaminoglycan hyaluronan (HA) are among these signals. HA is synthesized by transmembrane synthases and is composed of multiple disaccharide units of glucuronic acid and *N*-acetylglucosamine. HA molecules range in size from $\leq 2.5 \times 10^5$ Da to $\geq 4 \times 10^6$ Da. Different molecular weight forms of HA have distinct functions in the nervous system including the regulation of cell motility, growth, and differentiation.^{10,11} We found that HA accumulates coincident with astrogliosis in demyelinating MS lesions,¹² traumatic spinal cord injuries,¹³ perinatal white matter injuries,¹⁴ and lesions associated with vascular cognitive impairment¹⁵ and during normal aging.¹⁶ HA blocks OPC maturation and remyelination in lysolecithin-induced demyelinating lesions, suggesting that HMW HA accumulation contributes to remyelination failure.¹²

We reasoned that a strategy to promote remyelination within demyelinating lesions is to degrade accumulated HMW HA using hyaluronidases. During inflammatory responses outside the CNS, activated fibroblasts or other cells secrete hyaluronidases that degrade HA, generating HA digestion products that act as immune regulators.¹⁷ Reactive oxygen species at sites of inflammation further promote this degradation.¹⁸ Although hyaluronidases are expressed in the CNS,^{19,20} it is unclear whether HA is similarly degraded at extracellular sites of CNS inflammation where astrocytes are the principle source of HA.²¹

Paradoxically, we and others have found that the digestion of HMW HA by some hyaluronidases prevents OPC maturation. A recent study found that treatment of OPCs with HA degraded by a combination of hyaluronidases and β -glucuronidase blocked OPC maturation in vitro through a mechanism involving toll-like receptor-2 (TLR2).²⁰ This study also demonstrated that lower molecular weight forms of HA accumulate in MS lesions, that OPCs in vitro express multiple hyaluronidases, and that a broad spectrum hyaluronidase inhibitor can promote OPC maturation in vitro. The focus of the present study was to determine whether hyaluronidases are expressed in human and rodent demyelinating lesions; whether specific hyaluronidases alone can block OPC maturation; and whether blocking hyaluronidase activity can promote remyelination in vivo.

Here, we report that an extracellular hyaluronidase, called PH20, is elevated in rodent and human demyelinating lesions. We demonstrate that PH20 expression is elevated in OPCs and astrocytes in chronic demyelinated MS lesions and in acute lesions in mice with experimental autoimmune encephalomyelitis (EAE). Elevated expression of PH20, but not other hyaluronidases, is suf-

ficient to block OPC maturation. Digestion products of PH20, but not those generated by another hyaluronidase, potentially block remyelination in vivo. Furthermore, inhibiting HA synthesis does not influence OPC maturation, but blocking hyaluronidase activity promotes OPC maturation and remyelination. Collectively, these data suggest that PH20 is a potential therapeutic target to promote remyelination.

Materials and Methods

Reagents

HMW HA (100 μ g; 1.59×10^6 Da; Seikagaku Corporation, Tokyo, Japan) was dissolved in sterile phosphate-buffered saline (PBS), and HA fragments were generated with addition of bovine testicular hyaluronidase (BTH; Sigma, 100U/ml), *Streptomyces* hyaluronidase (StrepH; Sigma, St Louis, MO; 1-10U/ml), or PBS vehicle for 1 hour at 37°C, then incubated at 95 to 100°C for 30 minutes to heat inactivate enzymes. Digestions were evaluated by electrophoresis on a 0.5% agarose gel, followed by detection of HA using the cationic dye Stains-All (Sigma) as previously described.²² 4-Methylumbelliferone (4-MU; Sigma) was dissolved in PBS at 37°C and added to cultures at a final concentration of 0.1 to 1mM. 6-O-Palmitoyl-L-ascorbic acid (VCPAL; Sigma) was dissolved in dimethylsulfoxide at a concentration of 100mM and further diluted to a working concentration of 2.5 to 25 μ M for cell culture experiments and for coinjection into lysolecithin lesions. Turbidity assays for VCPAL activity and IC₅₀ calculations were performed as previously described.²³

Analysis of HA Size and Concentration

HA concentration, size distribution, and weight-average molar mass were determined by size exclusion chromatography–multi-angle laser light scattering (SEC-MALLS) as previously described²⁴ using PL Aquagel-OH 60, 40, and 20 (Polymer Laboratories, Church Stretton, UK) size exclusion columns in series. MALLS analyses of the SEC column eluate was performed in line using a Dawn DSP Laser Photometer in series with an Optilab DSP Interferometric Refractometer (both from Wyatt Technology Corporation, Santa Barbara, CA). Data were analyzed using ASTRA version 4.73 (Wyatt Technology Corporation), a dn/dc value of 0.153ml/g, and first-order Berry fits.

Lentiviral Construction and Infections

The expression vector (lentiviral vector–intron–green fluorescent protein) used for cloning is from Gregory A. Dissen (Oregon National Primate Research Center, Beaverton, OR).²⁵ PH20 and *Hyal5* cDNAs were obtained from Stephan Reitingier (Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, Austria). The *Hyal1* cDNA was from Barbara L. Triggs-Raine (University of Manitoba, Winnipeg, Manitoba, Canada).²⁶ *Hyal2* was obtained by real time polymerase chain reaction (RT-PCR) using the forward primer 5'-GAGTTCCTGAGCTGCTACCA-3' and the reverse primer 5'-AGGGGGAGAGATCCCTCATA-3'. The open reading frame

of PH20, *Hyal1*, *Hyal2*, and *Hyal5* were cloned in front of the cytomegalovirus promoter of a vector plasmid and packaged into a third-generation lentiviral vector. Cells were plated at 4 to 5×10^4 cells per coverslip and infected overnight using 2.5 to 5.0×10^5 transforming units (Multiplicity of infection (MOI) 1:50–1:100).

Cell Culture

Neural stem cells were isolated from the medial and lateral ganglionic eminences of embryonic day 13.5 mouse (C57BL/6) embryos and expanded in epidermal growth factor and fibroblast growth factor-2 (both at 10ng/ml) as neurospheres for 1 week as previously described.²⁷ To generate OPCs, neurospheres were dissociated into single cells in trypsin (0.05%; Invitrogen, Carlsbad, CA), washed in Dulbecco modified Eagle medium (DMEM) plus 10% fetal bovine serum and plated at 5×10^6 cells/ml on uncoated polystyrene plates in DMEM/F12 media containing 0.1% bovine serum albumin (BSA), platelet-derived growth factor (PDGF) AA and fibroblast growth factor-2 at 20ng/ml each, B27 supplement minus vitamin A (Gibco, Grand Island, NY), N1 supplement (Sigma), and D-biotin (10nM, Sigma). Small adherent oligospheres formed and were passaged once a week after dissociation with Accutase (Invitrogen). After 2 to 3 weeks, oligospheres were transferred to poly-L-ornithine-coated 100mm dishes. After 1 to 2 passages, highly enriched populations (>95%) of PDGF receptor (PDGFR)- α^+ Olig2 $^+$ O4 $^-$ OPCs (as assayed by immunocytochemistry; see Supplementary Fig 1 for example) were obtained and further propagated for in vitro experiments. For maturation experiments, OPCs were plated at 4 to 5×10^4 cells per coverslip and differentiated in DMEM/F12, 0.1% BSA, plus triiodothyronine (T3; 30nM, Sigma) and N-acetyl-L-cysteine (NAC, Sigma) as previously described.²⁷

Lysolecithin Lesions

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Oregon Health and Science University. Demyelination was induced in the rostral corpus callosum of 3- to 4-month-old C57BL/6J mice by injection of lysolecithin (4% in PBS; Sigma) as previously described¹² mixed with either vehicle (PBS), HMW HA, degraded HA, or VCPAL. Five days after lysolecithin injection, the same volumes of PBS, HA, or VCPAL were reinjected. Brains were harvested at 8 days after lysolecithin injection, fixed, and processed for immunohistochemistry as previously described.¹²

Induction of EAE

EAE was induced in female C57BL/6J mice as previously described.¹² Animals were anesthetized with isoflurane and perfused transcardially with 100U/ml sodium heparin (Sigma-Aldrich, St Louis, MO) containing saline followed by 4% paraformaldehyde in PBS. Spinal cords were dissected and processed for immunohistochemistry as described below.

Immunohistochemistry

Cells were fixed for 30 minutes at room temperature in 4% paraformaldehyde and washed 3 $\times$ in PBS. Lumbar spinal cord

tissue from mice with EAE was immersion fixed for 12 to 16 hours in 4% paraformaldehyde at 4°C, rinsed 3 $\times$ in PBS at room temperature, then cryoprotected in 30% sucrose overnight at 4°C. Tissues were embedded in optimal cutting temperature medium, rapidly frozen on dry ice, and cryosectioned at a thickness of 10 to 12 μ m. Cells and tissues were preblocked in 10% heat-inactivated fetal bovine serum for 45 minutes. Primary antibodies were diluted in blocking buffer and cells or EAE tissue were incubated overnight at 4°C, rinsed in blocking buffer 3 $\times$, then incubated with the appropriate species-specific fluoroconjugated secondary antibodies (Alexa 546 or Alexa 488; Molecular Probes, Eugene, OR) for 45 minutes. Antibodies used were: rat anti-PDGFR- α (1:250; BD Pharmingen, San Jose, CA), mouse anti-O4 (1:500; Millipore, Billerica, MA), mouse anti-myelin basic protein (MBP; 1:1,000; Sternberger Monoclonal, Lutherville, MD), rabbit anti-GalC (1:100, Millipore), rabbit anti-glial fibrillary acidic protein (GFAP; 1:1,000; Dako, Carpinteria, CA), rabbit anti-microtubule-associated protein-2 (1:1,000, Millipore), and rabbit anti-Olig2 (1:500, Millipore). Polyclonal rabbit antisera to PH20 were generously provided by James Overstreet (PH20, 1:1,000) and Patricia DeLeon (msSPAM, 1:400). HA was visualized by probing cells or tissues with biotinylated HA-binding protein (HABP; 1:200, Seikagaku) followed by avidin-conjugated Cy3 (1:1,000, Molecular Probes). Myelin was visualized by a 30-minute incubation of EAE sections with FluoroMyelin (1:400 in PBS, Invitrogen) following primary and secondary antibody application. Cell nuclei were visualized by 4',6-diamidino-2-phenylindole (DAPI) staining (Hoeschst 33342; 1:15,000; Molecular Probes). Sections from mice with lysolecithin lesions were analyzed for MBP reactivity as previously described.¹²

The use of tissues from individuals with MS was approved by the Human Subjects Committee at the University of Washington. For the analysis of MS patient lesions, paraffin sections from 5 MS patients (mean duration of disease, 16.4 years; mean age, 57.6 years; Supplementary Table 1) were deparaffinized in xylene and then rehydrated in graded alcohols. Endogenous peroxidase activity was blocked with 0.3% (vol/vol) hydrogen peroxide in methanol before washing the slides in water. Slides were heated in citrate buffer (10mmol/l, pH 6.0) for 5 minutes in a microwave for antigen retrieval. Sections were then preblocked in 5% normal goat serum. Primary antibody incubation was the same as indicated above, except that rabbit anti-PH20 (J. Overstreet) was used at a 1:500 dilution. Following biotinylated goat antirabbit secondary antibody binding, the avidin-biotin-peroxidase complex technique (Vectastain ABC kit and NovaRED; Vector Laboratories, Burlingame, CA) was used for visualization according to the manufacturer's instructions. In separate experiments, sections were also double-labeled with anti-PH20 (1:300) and either anti-MBP (1:500), anti-O4 (1:400), or anti-GFAP (1:50, Dako) followed by fluorescence-conjugated secondary antibodies as above.

Image processing and cell counts were performed using Photoshop 3.0 (Adobe Systems, San Jose, CA) and ImageJ (NIH, Bethesda, MD), respectively. For cell counts, 10 fields were randomly selected, and at least 500 cells were counted per

coverslip (3 coverslips per group). Mean cell numbers and standard deviations were calculated for each group.

Quantitative Analysis of OPC Maturation in Lysolecithin-Induced Lesions

Serial tissue sections (50 μ m) were cut on a vibrating microtome (VTS-1000; Leica Microsystems, Bannockburn, IL). Tissue sections were double-labeled for CC-1 (mature OL marker) and PDGFR- α (OL progenitor marker) and counterstained with Hoechst 33324. Tissue sections were first treated for antigen retrieval (10 minutes at 95°C in 50mM Tris-ethylenediaminetetraacetic acid buffer, pH 9.0). Tissue was incubated at 4°C for 3 days with a mouse monoclonal antibody CC-1 (OP80; 1:200 in PBS with 0.1% triton X-100; Calbiochem, San Diego, CA) and a rabbit polyclonal antisera against PDGFR- α (1:500; in PBS with 0.1% triton X-100; a generous gift of Dr William Stallcup, Burnham Institute, La Jolla, CA). Sections were also stained with DAPI to identify cell nuclei. Cell counts within lysolecithin-induced lesions were performed using a $\times 40$ objective with a counting radicle mounted on a Leica DMRA epifluorescence microscope. Cell counts were done in 3 separate zones that corresponded to a central region of chronic demyelination (zone 1), an adjacent region of active remyelination (zone 2), and the most lateral zone, which was equivalent to normal surrounding white matter (zone 3). For each study, a minimum of 5 animals were analyzed, and counts were obtained from duplicate adjacent sections. Given the irregular shape of the lesions, it was not feasible to precisely define the boundaries of these 3 zones. Each zone displayed a unique pattern of nuclear cell density that allowed it to be readily distinguished from adjacent zones. Zone 1 typically had a very high density of cell nuclei, whereas zone 2 had a density that was intermediate between zones 1 and 3. Within each zone, cell counts were done in a standardized number of fields that maximized the number that could be defined: zone 1 (2 fields), zones 2 and 3 (6 fields each).

Compound action potential (CAP) recordings

Brains were rapidly removed and submerged in ice cold artificial cerebrospinal fluid (aCSF; 124mM NaCl, 5mM KCl, 1.25mM NaH₂PO₄, 26mM NaHCO₃, 1.3mM MgSO₄, 2mM CaCl₂, 10mM glucose, pH 7.4) saturated with 95% O₂/ 5% CO₂. A 400 μ m-thick coronal slice corresponding to Bregma coordinates 0.5 to 0.9 was cut on a vibratome (Leica), collected, and incubated in aerated aCSF at room temperature for 1 hour prior to recording.

For recording, the slice was transferred to a perfusion chamber mounted on an upright microscope (Zeiss, Thornwood, NY). The tract of the injection electrode was usually visible; however, injection sites were confirmed with subsequent histochemical analysis of the slice. Recording and stimulating electrodes were positioned in the corpus callosum on the side of the injection and then moved to the opposite side to obtain recordings of untreated axons. The bipolar stimulating electrodes were fashioned from Teflon-insulated tungsten wires (World Precision Instruments, Sarasota, FL) with tips positioned

approximately 0.3mm apart. The stimulating electrodes were connected to a stimulator (Grass S88; Grass Technologies, West Warwick, RI) and stimulation isolation unit (Grass Technologies). Stimulus intensity was adjusted to obtain a maximal response. Recordings were obtained using glass microelectrodes filled with aCSF with a resistance of 3 to 5 M Ω . Recordings were amplified and filtered at 10kHz. Recordings were obtained from 2 sites across the width of the corpus callosum at approximately 1.5mm from the stimulating electrode. Ten responses were averaged at each recording site. The magnitude of the CAP waveform was determined by measuring the maximum negative deflection with respect to a tangent drawn between the adjacent positive deflections. For each recording session, 3 animals per group (treated with VCPAL or vehicle) were analyzed.

RT-PCR and Quantitative RT-PCR

Total RNA was isolated from cells or tissues using TRIzol (Invitrogen) following the manufacturer's instructions. mRNA was reverse transcribed into cDNA using random hex or oligo d(t) primers and a reverse transcriptase kit (Promega, Madison, WI). mRNA sequences for each hyaluronidase were downloaded from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>), and primers for RT-PCR were designed manually (supplied by Integrated DNA Technologies, Coralville, IA). See Supplementary Table 2 for primers used. RT-PCR was performed using GoTaq or Superscript DNA transcriptase (Promega or Invitrogen) in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) following the manufacturer's protocols. RT-PCR reaction products were analyzed by electrophoresis on a 1% agarose gel, and amplicons were visualized following staining with ethidium bromide. quantitative RT-PCR (qRT-PCR) was performed using predesigned primer and probe sets (TaqMan Assays; Applied Biosciences, Carlsbad, CA; HYAL1, Mm00476206; HYAL2, Mm0477731; and PH20 [SPAM1], Mm00486329), with an AB 7900HT fast PCR system using SDS 2.4 software (Applied Biosciences). Data Analysis was performed using Excel (Microsoft, Redmond, WA).

Statistics

An unpaired 2-tailed *t* test was used to compare changes in anti-PDGFR- α and anti-MBP immunolabeled cells in in vitro experiments. One-way analysis of variance followed by Tukey multiple comparison test was used to assess changes in the percentages, relative to controls, of mature OLs in cultures treated with VCPAL and for the analysis of qRT-PCR experiments. A value of *p* < 0.05 was considered significant.

Results

A Specific Hyaluronidase Blocks OPC Maturation In Vitro

We previously found that HMW forms of HA accumulate in demyelinating lesions and that the presence of HA blocks OPC maturation and remyelination.¹² Sloane and coworkers²⁰ reported that HMW HA digested by a combination of a hyaluronidase and β -glucuronidase

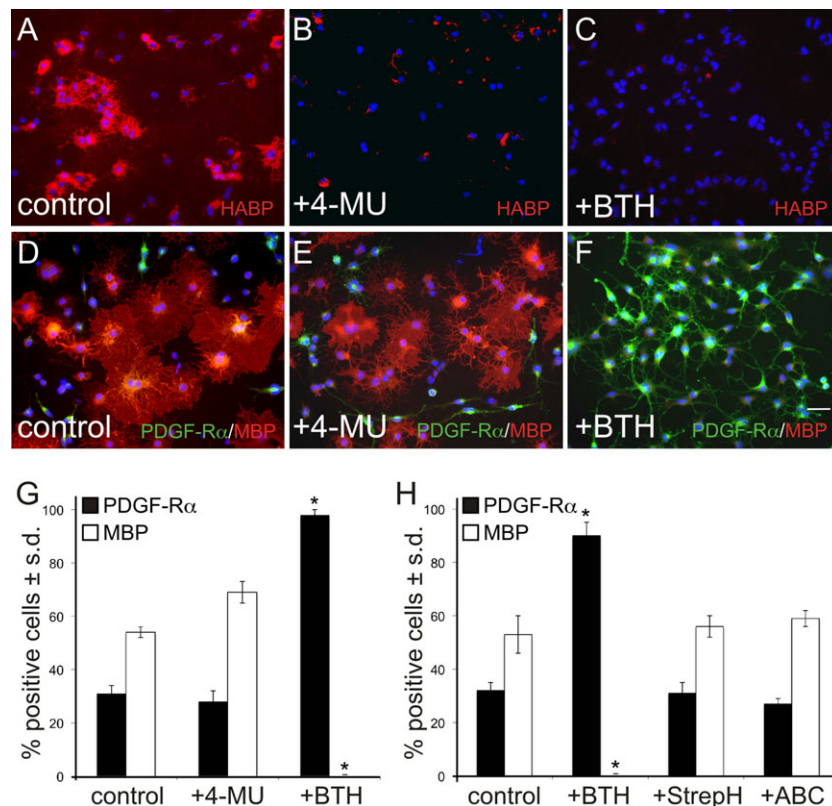


FIGURE 1: Bovine testicular hyaluronidase (BTH) inhibits oligodendrocyte progenitor cell (OPC) maturation in vitro. OPCs were grown in prodifferentiation medium containing triiodothyronine and N-acetyl-L-cysteine. Control OPC cultures (A, D) were analyzed relative to cultures treated with the hyaluronan (HA) synthase inhibitor 4-methylumbelliferone (4-MU; B, E) or with PH20 (C, F). After 96 hours, cultures were fixed and stained for the OL lineage markers myelin basic protein (MBP; red; to label mature OLs), platelet-derived growth factor receptor α (PDGFR- α ; green; to label OPCs), and 4',6-diamidino-2-phenylindole (blue; to visualize cell nuclei). (A–C) Representative levels of HA (as assayed using HA-binding protein [HABP] histochemistry) for each treatment group. (G) The total percentages of PDGFR- α ⁺ and MBP⁺ cells are quantified. PH20 treatment significantly inhibited OL maturation, as shown by a decreased percentage of cells expressing MBP compared to controls (<0.5% vs 54.1%) and an increased percentage of cells expressing PDGFR- α compared to controls (97.8% vs 30.9%). (E, G) Inhibiting HA synthesis did not significantly alter OL maturation. (H) Quantification of OPC maturation in the presence of chondroitinase ABC (ABC) or *Streptomyces*-derived hyaluronidase (StrepH). Unlike PH20, neither of these enzymes demonstrated any significant effect on OPC maturation. These experiments were performed 4 $\times$, with each data point assayed in triplicate wells. Scale bar = 10 μ m. * p < 0.0003. s.d. = standard deviation.

blocked OPC maturation in vitro. To further explore the role of HA in OPC maturation, we utilized a culture system in which mouse neural stem cells are differentiated in vitro into mouse OPCs (>90% PDGFR- α ⁺/O4[−]/GalC[−]/MBP[−] cells) that can subsequently generate pre-OLs (O4⁺/GalC[−]/MBP[−] cells) and mature OLs (MBP⁺ cells) in the presence of a prodifferentiation medium that contained T3 and NAC (see Supplementary Fig 1). After 3 hours in culture, we observed elevated pericellular HA synthesis by OPCs that increased over time (Supplementary Fig 2A–C). HA synthesis continued to accumulate in pre-OL cultures 24 hours following T3 and NAC treatment, but then diminished in cultures enriched with MBP⁺ cells, with HA remaining associated with cell bodies (see Supplementary Fig 2D, E). All 3 HA synthases were expressed by OPC cultures (see Supplementary Fig 2F).

Although as many as 60% of cells in these mouse OPC cultures became MBP⁺ OLs in prodifferentiation medium, approximately 40% of cells consistently failed to mature (Fig 1). We hypothesized that the low level of HA synthesized in our cultures was sufficient to block the maturation of a limited number of OPCs. We therefore tested whether the prevention of HA synthesis is sufficient to increase OL maturation in these cultures. To block HA synthesis, OPCs were switched to media containing the HA synthase inhibitor 4-MU. HA synthesis was almost completely blocked by 1mM 4-MU (see Fig 1A, B). Unexpectedly, we found that inhibiting HA synthesis had no significant effect on OL maturation compared to controls despite the nearly complete absence of HA in these cultures (see Fig 1D, E, G).

We next tested whether degradation of HA could influence OPC maturation. In contrast to the results of

Sloane and coworkers,²⁰ we found that BTH alone at concentrations between 25 and 100U/ml completely degraded HA in OPC cultures (see Fig 1C) and inhibited OPC maturation (see Fig 1F, G). This inhibitory activity was reversed by heat inactivation of the enzyme prior to HA digestion (data not shown). OPC maturation was not inhibited when cultures were treated with StrepH or with chondroitinase ABC (which degrades chondroitin sulfate into unsaturated disaccharides) at concentrations that were optimal for the digestion of HA and chondroitin sulfate, respectively (see Fig 1H). This finding is consistent with the hypothesis that specific HA digestion products are capable of inhibiting OPC maturation in vitro.

The PH20 Hyaluronidase, but Not Other Hyaluronidases, Potently Inhibits OPC Maturation

The soluble hyaluronidase activity in BTH has been attributed to the PH20 enzyme.²⁸ We therefore performed gain-of-function experiments to test how increased expression of PH20 and other mammalian hyaluronidases influenced OPC maturation. The cDNAs from mouse *Hyal1*, *Hyal2*, *Hyal5*, and *PH20* were cloned into a bicistronic lentiviral expression vector carrying the cDNA for enhanced green fluorescence protein (EGFP). OPCs were then infected with lentiviruses carrying each construct and analyzed for changes in OPC maturation as described above. OPC cultures infected with the control virus, carrying EGFP alone, demonstrated pericellular HA accumulation (Fig 2A), whereas HA was reduced in cultures infected with the *PH20* (see Fig 2B) and the *Hyal1*, *Hyal2*, and *Hyal5* viruses (data not shown). Cells infected with the *PH20*-carrying virus demonstrated a significant ($p < 0.0009$) and pronounced inhibition of OPC maturation as assessed by quantification of MBP⁺ cells, compared to cultures infected with a virus carrying only the EGFP cDNA (38% of control). Infection with the *Hyal1*-bearing virus had no significant effect on OL maturation, and cells infected with viruses carrying *Hyal2* and *Hyal5* only weakly inhibited OL maturation (approximately 80% and 73% of control, respectively; $p < 0.02$).

HA Digestion Products Generated by PH20 Are Sufficient to Inhibit Remyelination In Vivo

Taken together, our data support the hypothesis that specific HA digestion products generated by PH20 but not other hyaluronidases or chondroitinase potently inhibit OPC maturation. To determine whether PH20 generates distinct HA degradation products, we analyzed HMW HA that had been treated with either PH20 (BTH) or StrepH using the quantitative and sensitive SEC-MALLS

technique to characterize the HA products in these 2 digests (Fig 3A). The weight-average sizes of the PH20 (63.9 ± 0.5 kDa) and StrepH (2.4 ± 0.4 kDa) digests were very different. More importantly, the 2 distributions of HA product sizes in the 2 digests were essentially not overlapping. Only about 2% of the products in both digests were in the same size range (eg, 98% of the PH20 products ranged from 5.5 to 227kDa, and 98% of the StrepH products were <4.4 kDa).

We next determined whether the HA digestion products generated by PH20 activity were capable of blocking remyelination. We induced focal demyelination in the corpus callosum of mice using lysolecithin as previously described.¹² After 5 days, a second injection of either vehicle, HMW HA that had been incubated with vehicle, PH20-degraded HMW HA, or StrepH-degraded HMW HA was delivered into the original lesion site. Brains from these animals were analyzed 8 days after the initial lysolecithin injection ($n \geq 6$ per group). OPC maturation was assessed by analysis of MBP immunoreactivity in sections through lesions as previously described.¹² As expected, elevated MBP immunoreactivity at the sites of lesions was observed in animals injected with vehicle (Fig 3B). Consistent with our previous findings,¹² MBP expression was reduced in lesions injected with HMW HA (Fig 3C shows typical example). MBP expression was also reduced in lesions injected with PH20-digested HA, but was comparable to vehicle-injected controls in animals treated with StrepH-digested HA.

To further characterize and quantify the effects of PH20-digested HA on OPC maturation, we analyzed the proportion of cells within lesions (zone 1), at lesion borders (zone 2), and in adjacent unaffected white matter (zone 3) that express PDGFR- α and CC1 (a marker of mature OL cell bodies) in lysolecithin-induced demyelinated lesions (Supplementary Fig 3) after 8 days as above. We observed a significant increase in PDGFR- α ⁺ and CC1⁺ cells within lesions compared to unaffected white matter (see Fig 3F, G), consistent with the idea that OPCs are recruited to demyelinating lesions and mature into OLs that remyelinate spared axons. Interestingly, we did not observe increased numbers of PDGFR- α ⁺ cells at lesion borders, but there was a significant increase in the numbers of CC1⁺ cells at borders. The lesion borders are similar to so-called shadow plaques where partial remyelination occurs. It is possible, therefore, that the increased numbers of OLs at the borders of lesions are the result of increased OPC maturation due to a lack of inhibitory signals in this location. Adding PH20-induced HA digestion products to lesions resulted in a significant increase in PDGFR- α ⁺ cells and a

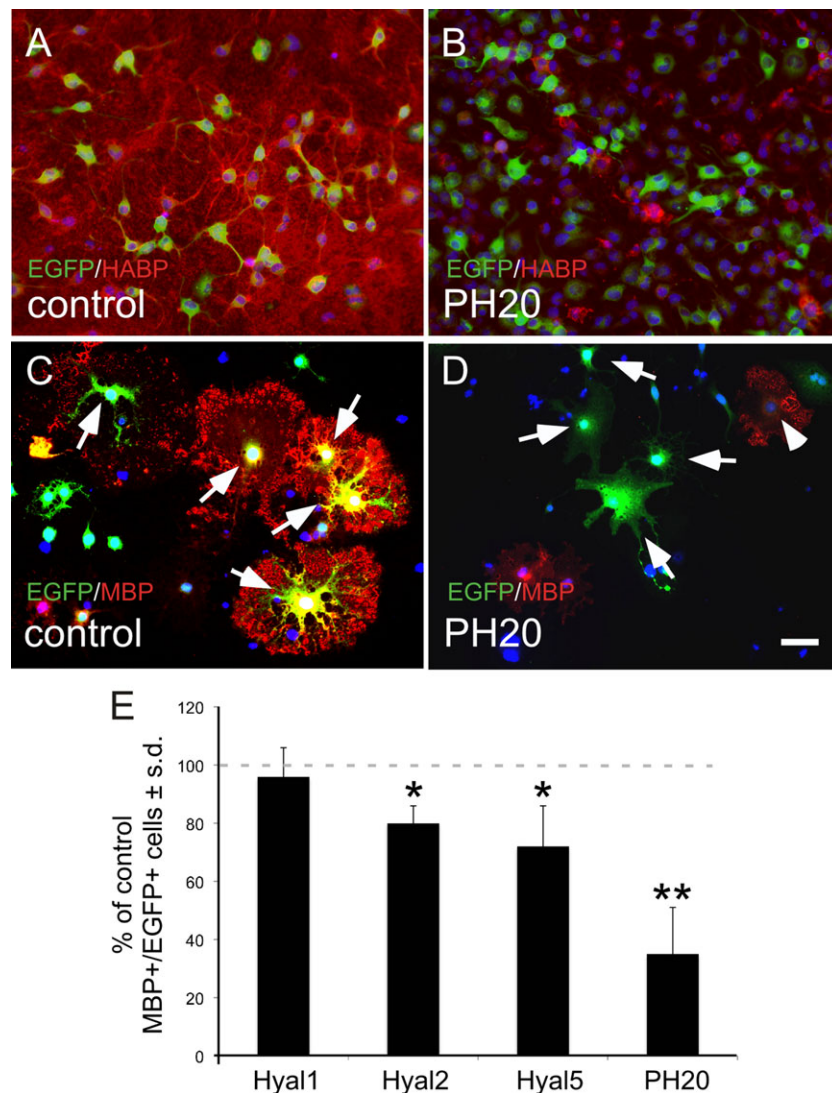


FIGURE 2: Elevated expression of PH20, the soluble hyaluronidase in PH20, blocks oligodendrocyte (OL) maturation in vitro. Oligodendrocyte progenitor cells were infected with lentiviruses carrying enhanced green fluorescence protein (EGFP) only (control; A, C) or viruses carrying EGFP plus *Hyal1*, *Hyal2*, *Hyal5* (not shown), or *PH20* (B, D). Cells were grown for 96 hours and stained for EGFP (green) and either hyaluronan-binding protein (HABP; A, B; red) or myelin basic protein (MBP; C, D; red). (E) Quantification of MBP expression by EGFP⁺ cells relative to cultures infected with the virus carrying EGFP alone. PH20 overexpression resulted in reduced HABP staining and inhibited OL maturation compared to cultures infected with only EGFP. *HYAL2* overexpression partially inhibited OL maturation (78.48% of control). *HYAL5* overexpression also partially inhibited OL maturation (70.08% of control). These experiments were performed 4× with each data point assayed in triplicate wells. **p* < 0.02, ***p* < 0.0009. Scale bar = 10 μm. s.d. = standard deviation.

significant decrease in CC1⁺ cells (see Fig 3F, G), indicating that OPCs were still recruited to lesions but failed to mature into OLs. Specific HA digestion products produced by PH20 are therefore capable of blocking OL maturation in demyelinated lesions in vivo.

OPCs Express Hyaluronidases and Degrade HA

A previous study using only immunohistochemistry with single antibodies reported that OPCs in vitro express multiple hyaluronidases, including PH20.²⁰ Other reports, however, had indicated that PH20 was localized

to testes but not other tissues.^{29–32} To confirm that PH20 is expressed by OPCs, we isolated total RNA from mouse testes (as a positive control for testicular hyaluronidases), from OPCs grown in vitro, and from adult mouse corpus callosum, then performed RT-PCR using primers specific for the hyaluronidases with known hyaluronidase activity in mouse testes (*Hyal1*, *Hyal2*, *Hyal5*, and *PH20*). We found that *Hyal1*, *Hyal2*, and *PH20* but not the testes-specific *Hyal5* were expressed by OPCs and in white matter using both RT-PCR (Fig 4) and qRT-PCR (data not shown). *Hyal1*, *Hyal2*, and *PH20*

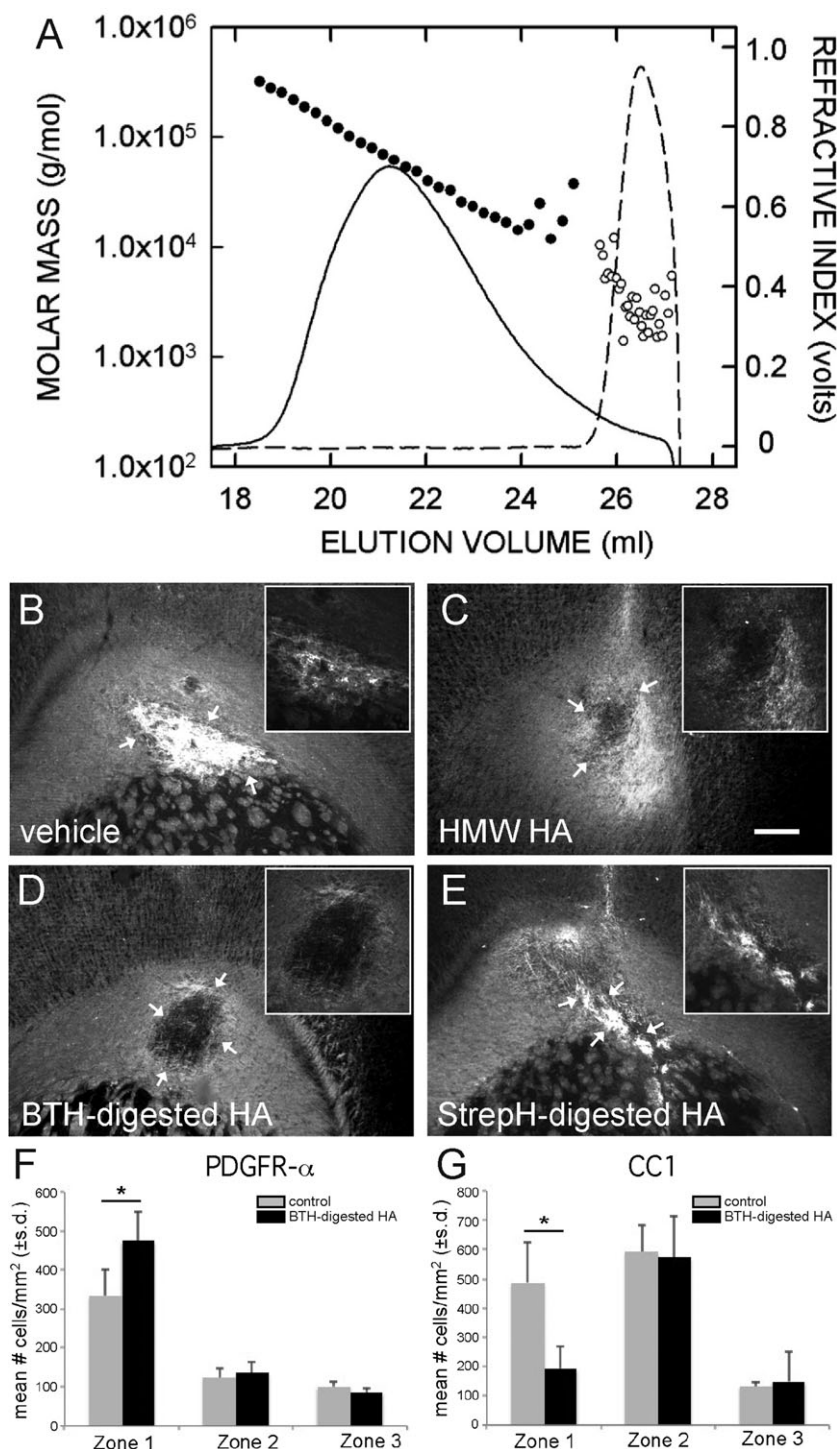


FIGURE 3: PH20 digestion products of hyaluronan (HA) block remyelination. (A) HA samples were treated with bovine testicular hyaluronidase (BTH)/PH20 (solid line and black circles) or *Streptomyces* hyaluronidase (StrepH; dashed line and white circles) and analyzed by size exclusion chromatography–multiangle laser light scattering to determine the amount (refractive index, solid lines and dashed lines) and the molar masses based on light scattering (black circles and white circles) of HA products present. The weight-average masses of the BTH/PH20 and StrepH samples were 63.9 ± 0.5 kDa and 2.4 ± 0.4 kDa, respectively. Overlap of the 2 digests was $\leq 2\%$ (98% of the BTH/PH20 products ranged from 5.5 to 227 kDa and 98% of the StrepH products were < 4.4 kDa). (B) Vehicle alone, (C) high molecular weight (HMW) HA (100 μ g/ml), (D) BTH/PH20-degraded HA (100 μ g/ml), or (E) StrepH-degraded HA (100 μ g/ml) was stereotactically coinjected with lysolecithin into the corpus callosum. Each HA preparation was reinjected without lysolecithin into demyelinated lesions 5 days later. Remyelination was determined by immunolabeling for myelin basic protein (MBP) 8 days after lysolecithin injection. Arrows in B and E indicate the typical pattern of enhanced MBP immunoreactivity (white) in areas of apparent remyelination. Note that because of the broad dynamic range of the signal, areas of remyelination appear overexposed. Insets show digitally enlarged images of lesions with reduced brightness and contrast, demonstrating the morphology within lesions. Arrows in C and D indicate regions of persistent lysolecithin-induced demyelination. This experiment was performed $3\times$. Scale bar = 250 μ m. (F) Quantification of the density of platelet-derived growth factor receptor α (PDGFR- α)⁺ cells within lesions (zone 1), at lesion borders (zone 2), and in adjacent unaffected white matter (zone 3). Note that there is an increase in PDGFR- α ⁺ cells within lesions that is enhanced by PH20-digested HA. * $p < 0.002$. (G) Quantification of CC1⁺ cells in the different zones of demyelinated lesions. Note that CC1⁺ cells increase in number both within lesions and at lesion borders. PH20-digested HA significantly inhibits the maturation of these mature OLs within the lesion. * $p < 0.001$. s.d. = standard deviation.

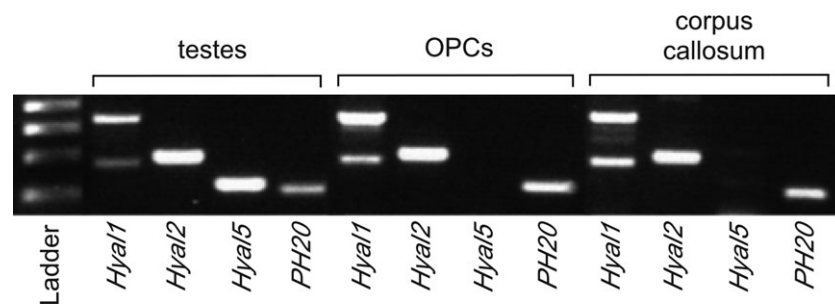


FIGURE 4: Oligodendrocyte progenitor cells (OPCs) express multiple hyaluronidases. Total mRNA was isolated from mouse testes (as a positive control), OPCs, and adult mouse corpus callosum, reverse transcribed, and subjected to real time polymerase chain reaction. OPCs express *Hyal1*, *Hyal2*, and *PH20* but not the testes-specific *Hyal5*. The same patterns of hyaluronidase expression were found in the corpus callosum.

transcripts were also amplified from RNA isolated from whole brain, cortex, and spinal cord (data not shown). To verify that the transcripts we had amplified by RT-PCR were indeed *PH20*, 3 distinct sets of primers (see Supplementary Table 2) were used to amplify separate regions of *PH20* mRNA isolated from adult mouse brain and were sequenced, confirming that OPCs express bona fide *PH20* RNA (data not shown).

Given that *PH20* transcripts are expressed by OPCs and in the corpus callosum, and that elevated *PH20* expression is sufficient to inhibit OPC maturation, we examined *PH20* expression in OL lineage cells at different stages of differentiation. *PH20* protein expression was assayed in proliferating OPCs and maturing OLs by immunocytochemistry using 2 separate *PH20* antibodies (generous gifts of P. DeLeon and J. Overstreet) in combination with the OL lineage-specific markers *PDGFR- α* ,

O4, or *MBP*. *PH20* immunostaining localized both to the cell body and processes of immature *PDGFR- α* ⁺ OPCs (Fig 5A–D). In contrast, *PH20* was highly enriched in the cell body and in the tips of processes in *O4*⁺ pre-OLs (see Fig 5E–H). *PH20* expression was reduced in mature *MBP*⁺ OLs, where it became restricted to the cell body (see Fig 5I–L). Altogether, these data indicate that the distribution of *PH20* is maturation dependent and becomes reduced in processes and restricted to the cell body as OPCs mature into OLs.

Because *PH20* is a glycosyl-phosphatidylinositol-anchored hyaluronidase that can function at neutral pH, we reasoned that its expression by OPCs would lead to the digestion of extracellular HA. We therefore determined whether OPCs are capable of degrading HA. OPCs were plated onto coverslips uniformly coated with HMW HA (approximately 1.59MDa), differentiation

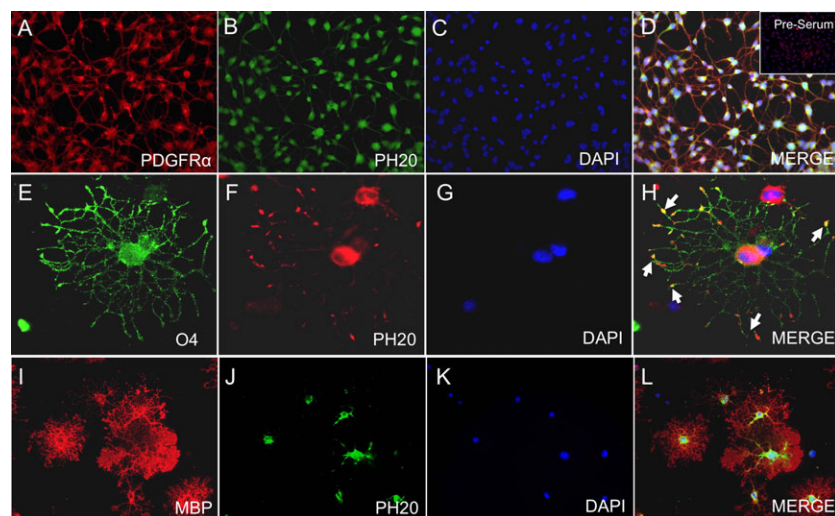


FIGURE 5: *PH20* expression is differentially localized on proliferating oligodendrocyte progenitor cells (OPCs) compared to mature oligodendrocytes (OLs) in vitro. *PH20* expression was confirmed by immunocytochemistry in OPCs (A–D), preoligodendrocytes (E–H), and maturing OLs (I–L). In OPCs, *PH20* expression (B; green) was seen in cell bodies and processes of platelet-derived growth factor α (*PDGFR α*)⁺ OPCs (A; red). (D) Inset shows lack of staining in OPCs incubated with preimmune serum. (F) Distribution of *PH20* (red) in an *O4*⁺ (E; green) cell. (H) *PH20* expression is observed in the tips of *O4*⁺ processes (arrows). *PH20* expression became confined to the cell body (J; green) of myelin basic protein (*MBP*)⁺ OLs (I; red). 4',6-Diamidino-2-phenylindole (DAPI; blue) was used to identify cell nuclei. Merged images are shown in D, H, and L.

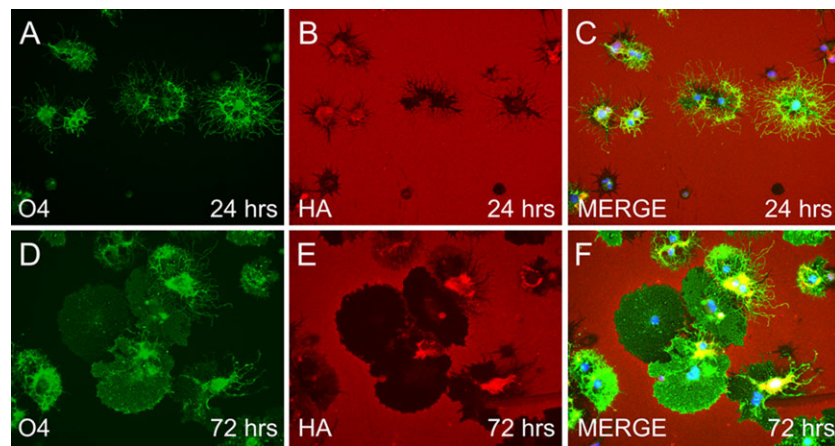


FIGURE 6: Oligodendrocyte progenitor cells (OPCs) and later oligodendrocyte (OL) stage cells degrade hyaluronan (HA). OPCs grown in the presence of triiodothyronine and N-acetyl-L-cysteine were plated onto coverslips coated with high molecular weight HA, allowed to mature for 24 (A–C) or 72 (D–F) hours, and stained with O4 (green), hyaluronan-binding protein (HABP; red), and 4',6-diamidino-2-phenylindole (blue). Pronounced degradation of HA, as assayed by loss of HABP staining, was seen around cell bodies and the extensive O4⁺ processes (A–C) at 24 hours. By 72 hours, substantial degradation of HA was also observed (D–F) in areas corresponding to the presence of O4⁺ membranes that resemble myelin sheets. Note that HA was preserved around many OPC and mature OL cell bodies.

was promoted in the presence of T3 and NAC for 24 or 72 hours, then cells were fixed and labeled with an anti-O4 antibody and a biotinylated HABP. We observed a loss of HABP staining around the cell bodies and processes of O4⁺ cells at 24 hours in vitro (Fig 6A–C). After 72 hours of OPC differentiation, O4 staining demonstrated membrane sheets of maturing OLs that were associated with a pronounced loss of HABP staining (see Fig 6D–F). Collectively, these data indicate that OL lineage cells express PH20 and are capable of digesting extracellular HMW HA.

PH20 Expression Is Elevated in Demyelinating Lesions

Because we found that HMW HA accumulates in chronic demyelinated lesions,¹² that PH20 expressed by OPCs can degrade HMW HA, and that PH20 breakdown products can inhibit OPC maturation and remyelination, we hypothesized that PH20 expression may be upregulated in demyelinated lesions. In mice with EAE, we observed that PH20 was not detectable in unaffected adult white matter but was elevated in active, acute demyelinated spinal cord lesions (eg, circled areas in Fig 7A–D), where it was expressed by both reactive astrocytes and occasionally by OPCs (data not shown).

In chronically demyelinated plaques of MS patients ($n = 5$; see Supplementary Table 1), PH20 immunoreactivity was enriched at the borders of lesions, with only small numbers of immunoreactive cells in the center of lesions (see Fig 7E, *arrows*; Supplementary Fig 4). Numerous cells expressed PH20 at the lesion borders. Double-labeling immunohistochemistry revealed that the

majority of these PH20⁺ cells originated from GFAP⁺-reactive astrocytes (see Fig 7G; *arrows*) as well as a subpopulation of O4⁺ preoligodendrocytes (see Fig 7H; *arrows*). HA staining was reduced in PH20-high areas in MS lesions, consistent with the finding that PH20⁺ OPCs can degrade extracellular HA (Supplementary Fig 5). These data indicate that PH20 expression and activity are elevated in rodent and human demyelinating lesions.

Pharmacological Inhibition of Hyaluronidase Activity Promotes OL Maturation and Functional Remyelination

Given that OPCs express multiple hyaluronidases and that degradation products of PH20 inhibit OPC maturation, we reasoned that blocking the activity of hyaluronidases, and therefore the generation of inhibitory HA breakdown products, would promote OL maturation. We chose to inhibit hyaluronidase activity in OPC cultures with the hyaluronidase inhibitor VCPAL for 72 to 96 hours. Consistent with previous studies, we found that VCPAL inhibited BTH/PH20 activity, with an IC₅₀ of 25 to 35 μ M (Supplementary Fig 6). We examined VCPAL-treated and control (vehicle) cultures for changes in the expression of the OL lineage markers PDGFR- α to label OPCs and MBP to label mature OLs. VCPAL treatment prevented HA degradation as assayed by HABP labeling (data not shown) and significantly increased the proportion of cells that became mature OLs, assayed as the total percentage of cells expressing MBP as compared to cultures treated with vehicle alone (Fig 8A–C).

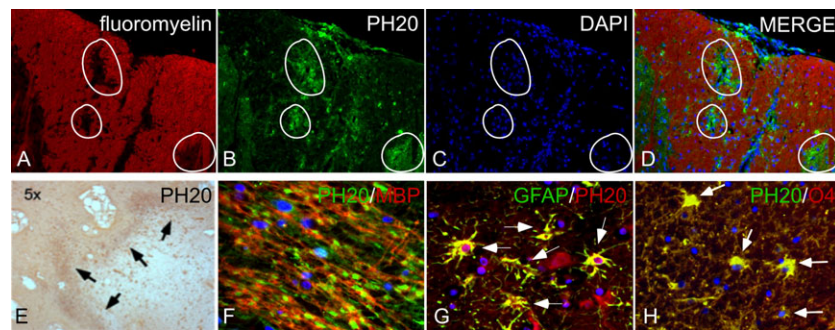


FIGURE 7: PH20 expression in demyelinated lesions. (A–D) Sections of lumbar spinal cord from mice with experimental autoimmune encephalomyelitis, 21 days postinoculation with myelin oligodendrocyte glycoprotein. PH20 immunoreactivity (green; B, D) was elevated in areas where there was demyelination (indicated by white circles), identified by loss of FluoroMyelin (red) staining (A, D) and increased 4',6-diamidino-2-phenylindole (DAPI) labeling (C, D). Merged image is shown in D. (E–H) PH20 is also expressed by glial cells at the borders of chronic, cortical multiple sclerosis (MS) lesions. (E) PH20 immunoreactivity was enriched at the borders of lesions (arrows). (F) Numerous cells expressed PH20 in the lesion borders (myelin basic protein [MBP], red; PH20, green). (G) Double-labeling of an MS patient's lesion with antibodies against PH20 (red) and glial fibrillary acidic protein (GFAP; green). (H) Double-labeling of MS lesions with PH20 (green) and O4 (red). Original magnification, $\times 5$ (A–E), $\times 40$, (F–H). Sections were counterstained with DAPI (blue) to label cell nuclei.

To assess whether inhibition of hyaluronidase activity is sufficient to promote OPC maturation *in vivo*, we coinjected VCPAL with HMW HA into lysolecithin-induced corpus callosum lesions. In animals with lysolecithin-induced lesions, VCPAL resulted in elevated MBP immunoreactivity (see Fig 8D, E; *arrows*) similar to that seen in animals injected with vehicle alone (eg, Fig 3B). When we quantified OPCs and mature OLs within lesions, at lesion borders, and in adjacent unaffected white matter as described above, we found that there was a trend toward increased numbers of CC1⁺ cells and decreased numbers of PDGFR- α ⁺ cells within lesions treated with HMW HA and VCPAL compared to controls injected with vehicle and HMW HA (see Fig 8F, G, zone 1). At lesion borders, VCPAL-treated animals demonstrated a significant increase in PDGFR- α ⁺ and CC1⁺ cells, indicating that VCPAL enhanced recruitment and maturation of OPCs, especially at lesion borders.

We next tested whether VCPAL could promote the functional remyelination of lysolecithin-induced lesions. Mice were treated as above with VCPAL or vehicle ($n = 3/\text{group/experiment}$), and slices of corpus callosum that included the injection site were analyzed using CAP recordings. Representative recordings of slices are shown in Figure 9A to C. There was a lack of fast latency peaks (representing myelinated axons) in animals injected with HA and vehicle, but increased high-latency peaks in animals that received VCPAL, similar to those observed in the control hemisphere. Following the recording, the 400 μm slices were fixed and immunolabeled as whole mounts for MBP. Immunolabeling verified increased MBP immunoreactivity in animals treated with VCPAL as compared to animals injected with vehicle (see Fig

9D, E). Quantification of high:low latency peak ratios (see Fig 9F) confirmed that VCPAL increased conduction velocities in the corpus callosum of mice treated with lysolecithin and HMW HA, whereas treatment with vehicle had no effect on conduction velocities.

Altogether, these findings support the hypothesis that the hyaluronidase activity of OPCs and other cells in demyelinating lesions contributes to the impairment of OL maturation and thus to the remyelination failure seen in chronically demyelinated lesions and MS plaques. These data further suggest that inhibiting hyaluronidase activity, and particularly PH20 activity, may be an efficacious strategy to promote remyelination.

Discussion

We have demonstrated for the first time that the PH20 hyaluronidase is elevated in astrocytes and OPCs in demyelinating lesions from MS patients and rodents with EAE; that elevated expression of PH20 by OPCs is sufficient to inhibit OPC maturation; that HA digestion products generated by PH20 are sufficient to inhibit OPC maturation and remyelination; and that inhibiting hyaluronidase activity promotes OPC maturation and remyelination *in vivo*. Although an earlier study reported that a wide range of HA molecules with different molecular weights are present in demyelinating MS lesions,²⁰ our findings support the hypothesis that the molecules that inhibit OPC maturation and remyelination are a specific set of digestion products generated by PH20.

Previous studies from our group and others reported that HMW HA accumulates in demyelinating

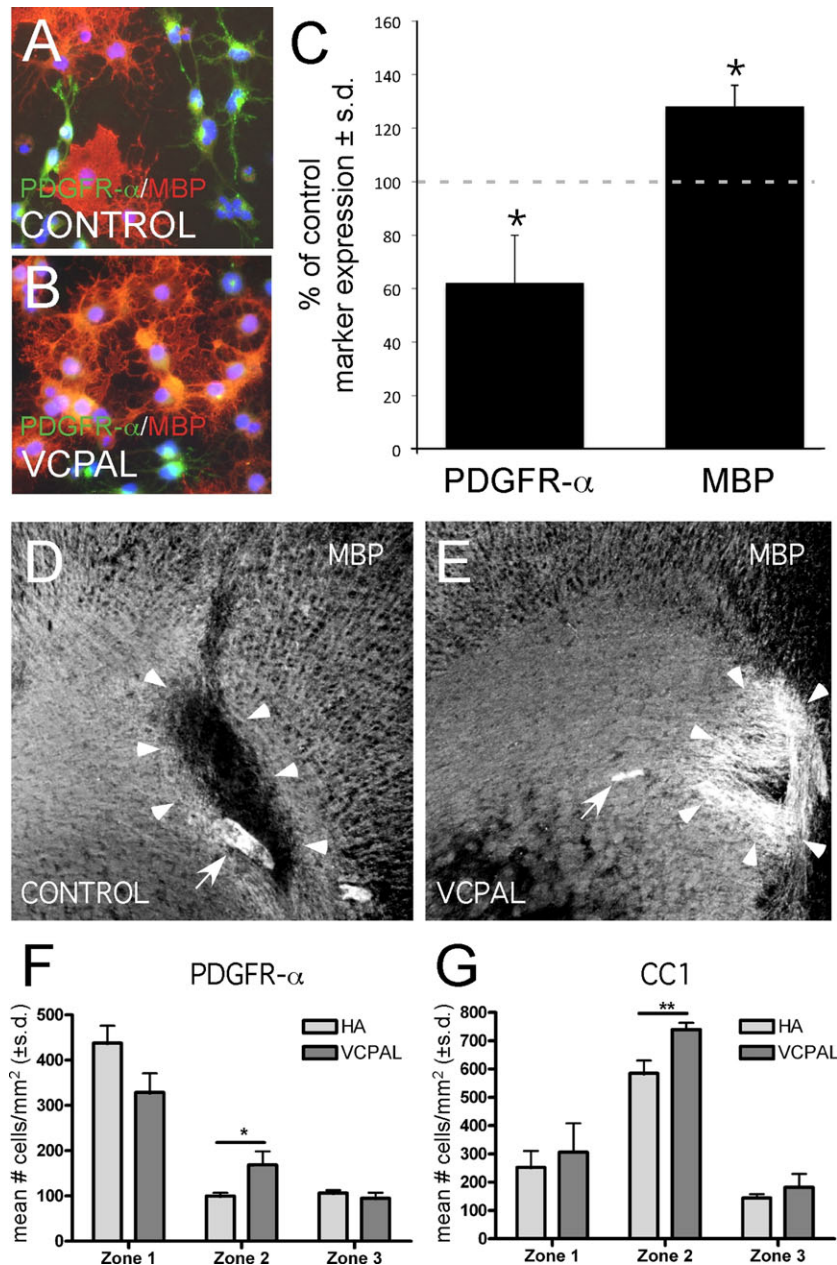


FIGURE 8: Inhibiting endogenous hyaluronidase activity promotes oligodendrocyte (OL) maturation in vitro and remyelination in vivo. Oligodendrocyte progenitor cells (OPCs) were grown in (A) OL differentiation medium alone or (B) with 25 μ M 6-O-palmitoyl-L-ascorbic acid (VCPAL) for 72 to 96 hours and stained with myelin basic protein (MBP), platelet-derived growth factor receptor α (PDGFR- α), and 4',6-diamidino-2-phenylindole. (C) Total MBP⁺ and PDGFR- α ⁺ cells are quantified. Consistent with the hypothesis that hyaluronidases expressed by OPCs generate hyaluronan (HA) digestion products that inhibit OL maturation, blocking HYAL activity with VCPAL increased the total percentage of MBP⁺ cells compared to controls (69% vs 54% $p = 0.005$) while decreasing the total percentage of PDGFR- α ⁺ cells (19% vs 30% in control, $p = 0.02$). (D, E) MBP immunoreactivity (white) of lysolecithin lesions treated with (D) high molecular weight (HMW) HA and vehicle or (E) HMW HA with 25 μ M VCPAL. Arrowheads indicate the borders of the demyelinated lesion in D and the area of remyelination in E; arrows indicate tissue areas with autofluorescence. Scale bar = 250 μ m. (F) Quantification of PDGFR- α ⁺ cells within lesions (zone 1), at lesion borders (zone 2), and in adjacent unaffected white matter (zone 3). Note that there was a trend within lesions for a reduction in the number of PDGFR- α ⁺ cells. Within the lesion border, there was a significant increase in OL progenitors. (G) Quantification of CC1⁺ cells within the different lesion zones. The apparent recruitment of OL progenitors in the lesion border (F) was accompanied by an increased density of CC1⁺ mature OLs in the lesion border in G (and a similar trend within lesions). ** $p < 0.001$. s.d. = standard deviation.

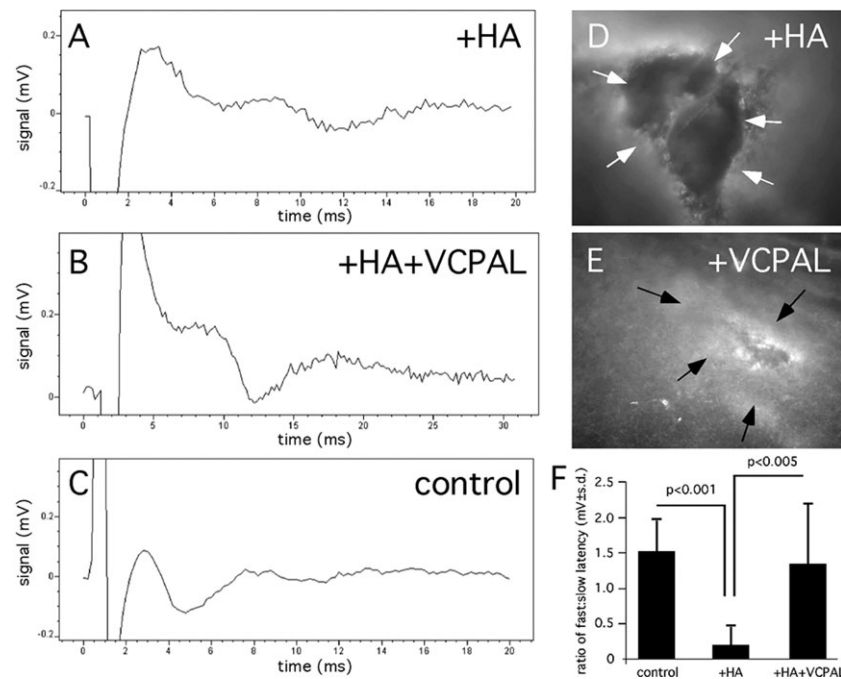


FIGURE 9: Compound action potential recordings of lysolecithin-induced demyelinating lesions treated with 6-O-palmitoyl-L-ascorbic acid (VCPAL) or vehicle. (A–C) Raw traces of recordings of slices from (A) a lesion treated with vehicle and HA; (B) a lesion treated with hyaluronan (HA) and 25 μ M VCPAL; and (C) the uninjected contralateral hemisphere of the animal in A. Following recording, slices were fixed in paraformaldehyde, then stained as whole mounts with an anti-myelin basic protein antibody (white). (D) A slice from an animal injected with HA and vehicle. (E) A slice from an animal injected with HA and VCPAL. (F) Quantification of the ratios of averaged recordings of fast and slow latencies. Note that slices treated with VCPAL demonstrated significant recovery of fast latency transduction. s.d. = standard deviation.

lesions and that adding HMW HA to demyelinated lesions prevents OPC maturation and remyelination.^{12,20} HA and HA receptors, including CD44 and TLR2, are elevated coincident with astrogliosis following a variety of CNS insults including demyelinating lesions in MS patients and mice with EAE,^{12,20} traumatic spinal cord injuries,¹³ and ischemia.³³ HA is also elevated in the brains of aged rodents³⁴ and nonhuman primates,¹⁶ and in patients with Alzheimer disease and age-related vascular brain injury.^{15,35,36} Myelination disturbances occur in each of these conditions. Our data support a model for remyelination failure whereby HMW HA is synthesized by reactive astrocytes and other cells within CNS lesions, the HA is degraded into specific HA digestion products by PH20 expressed by glial cells in the lesion microenvironment as well as OPCs recruited to lesions, and these HA digestion products in turn inhibit OPC maturation. The HMW HA added to demyelinating lesions in previous studies was therefore likely digested by PH20 expressed within lesions, and these digestion products and not HMW HA itself blocked remyelination. Thus, inhibiting hyaluronidase activity and blocking signaling by hyaluronidase-generated HA digestion products are potentially efficacious strategies for promoting remyelination in numerous conditions.

Our finding that PH20 expression in OPCs is localized to the tips of cell processes and then is restricted to the cell body in more differentiated OLs suggests that PH20 may play a role in regulating OPC–axon interactions during myelination. Thus, although PH20 digestion products inhibit OPC maturation and remyelination, and blocking PH20 activity promotes OPC maturation in vitro, the enhanced remyelination we observed following treatment with a hyaluronidase inhibitor could be the result of altering the regulated interactions between OPC processes and axons. Additional studies will be required to test this hypothesis.

HA digestion products may influence OPC maturation through a number of mechanisms. A study in *Xenopus* tadpoles demonstrated that glycogen synthase kinase-3b (GSK3 β), a serine/threonine protein kinase that is part of the Wnt signaling cascade, is activated by HA signaling.³⁷ The Wnt signaling cascade and GSK3 β in particular have been implicated in regulating OPC maturation.^{38–41} Inhibition of GSK3 β stimulates remyelination in adult mice.⁴⁰ It is possible therefore that HA digestion products inhibit OPC maturation at least in part through the activation of GSK3 β . HA digestion products may also signal through TLR2 or TLR4,^{42–46} both of which can also influence GSK3 β activation.^{47,48} Toll-like

receptors are expressed by OPCs, and 1 study has suggested that HA-mediated inhibition of OPC maturation is dependent on TLR2.²⁰ The contributions of other HA receptors, including CD44 and the receptor for hyaluronan-mediated motility, in regulating OPC maturation have yet to be elucidated.

HA digestion products likely act in concert with other signals in demyelinated lesions to prevent remyelination. Like HA accumulation, many of these signals appear to be linked to reactive astrogliosis. Reactive astrocytes in spinal cord injuries increase their expression of bone morphogenetic proteins that inhibit OPC differentiation with concurrent promotion of astrocyte differentiation.⁴⁹ It is possible that HA, which is synthesized predominantly by reactive astrocytes, acts on OPCs following its digestion by PH20 in concert with these and other inhibitory mechanisms including the Nogo receptor-interacting protein LINGO-1 to prevent remyelination.⁵⁰

Although we have demonstrated that inhibiting hyaluronidase activity is sufficient to promote remyelination in lesions where HA accumulates, such broad-acting inhibitors are not likely to be useful as therapeutic agents. Hyal1 and Hyal2, for example, are widely distributed in many different tissues.⁵¹ *Hyal1*-null mice develop osteoarthritis,⁵² and humans with *HYAL1* mutations develop a lysosomal storage disorder, mucopolysaccharidosis IX.⁵³ *Hyal2*-null mice develop skeletal and hematological abnormalities.⁵⁴ In contrast, although PH20 mRNA has been detected at high levels in testis and in low abundance in a limited number of other tissues, *PH20*-null mice do not display any significant pathological phenotypes.⁵⁵ Our finding that PH20 is specifically upregulated in demyelinating lesions and is sufficient to block OPC maturation suggests that agents that target PH20 and not other hyaluronidases may be both safe and efficacious as long-term therapies for the promotion of remyelination.

Acknowledgment

This work was supported by NIH (NINDS) grants NS056234 (L.S.S.), NS054044, and NS045737-06S1/06S2 (S.A.B.), core grant RR00163 (L.S.S.) supporting the Oregon National Primate Research Center, and grant P30-NS061800 (L.S.S.) supporting the imaging core at the Oregon National Primate Research Center; grant SRA-10-055 from Fast Forward, LLC (L.S.S.), grant W81XWH-10-1-0967 from the Congressionally Directed Research Programs Multiple Sclerosis Research Program (L.S.S., P.H.W.); the Laura Fund for Multiple Sclerosis Research (M.P.); and grants from the March of Dimes

Birth Defects Foundation and the American Heart Association (S.A.B.).

We thank Drs P. DeLeone and J. Overstreet for PH20 antibodies and L. Sacha for administrative assistance.

Potential Conflicts of Interest

Nothing to report.

References

- Franklin RJ, Ffrench-Constant C. Remyelination in the CNS: from biology to therapy. *Nat Rev Neurosci* 2008;9:839–855.
- Wolswijk G. Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. *J Neurosci* 1998;18:601–609.
- Scolding N, Franklin R, Stevens S, et al. Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis. *Brain* 1998;121(pt 12):2221–2228.
- Chang A, Nishiyama A, Peterson J, et al. NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *J Neurosci* 2000;20:6404–6412.
- Maeda Y, Solanky M, Menonna J, et al. Platelet-derived growth factor- α receptor-positive oligodendroglia are frequent in multiple sclerosis lesions. *Ann Neurol* 2001;49:776–785.
- Chang A, Tourtellotte WW, Rudick R, Trapp BD. Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. *N Engl J Med* 2002;346:165–173.
- Wolswijk G. Oligodendrocyte precursor cells in the demyelinated multiple sclerosis spinal cord. *Brain* 200;125(pt 2):338–349.
- Kotter MR, Stadelmann C, Hartung HP. Enhancing remyelination in disease—can we wrap it up? *Brain* 2011;134(pt 7):1882–1900.
- Kremer D, Aktas O, Hartung HP, Küry P. The complex world of oligodendroglial differentiation inhibitors. *Ann Neurol* 2011;69:602–618.
- Sherman LS, Back SA. A ‘GAG’ reflex prevents repair of the damaged CNS. *Trends Neurosci* 2008;31:44–52.
- Preston M, Sherman LS. Neural stem cell niches: roles for the hyaluronan-based extracellular matrix. *Front Biosci (Schol Ed)* 2011;3:1165–1179.
- Back SA, Tuohy TM, Chen H, et al. Hyaluronan accumulates in demyelinated lesions and inhibits oligodendrocyte progenitor maturation. *Nat Med* 2005;11:966–972.
- Struve J, Maher PC, Li YQ, et al. Disruption of the hyaluronan-based extracellular matrix in spinal cord promotes astrocyte proliferation. *Glia* 2005;52:16–24.
- Buser JR, Maire J, Riddle A, et al. Arrested pre-oligodendrocyte maturation contributes to myelination failure in premature infants. *Ann Neurol* 2012;71:93–109.
- Back SA, Kroenke CD, Sherman LS, et al. White matter lesions defined by diffusion tensor imaging in older adults. *Ann Neurol* 2011;70:465–476.
- Cargill R, Kohama SG, Struve J, et al. Astrocytes in aged nonhuman primate brain gray matter synthesize excess hyaluronan. *Neurobiol Aging* 2012;33:830.e13–830.e24.
- Jiang D, Liang J, Noble PW. Hyaluronan as an immune regulator in human diseases. *Physiol Rev* 2001;91:221–264.
- Soltés L, Mendichi R, Kogan G, et al. Degradative action of reactive oxygen species on hyaluronan. *Biomacromolecules* 2006;7:659–668.

19. Al'Qteishat A, Gaffney J, Krupinski J, et al. Changes in hyaluronan production and metabolism following ischaemic stroke in man. *Brain* 2006;129(pt 8):2158–2176.
20. Sloane JA, Batt C, Ma Y, et al. Hyaluronan blocks oligodendrocyte progenitor maturation and remyelination through TLR2. *Proc Natl Acad Sci U S A* 2010;107:11555–11560.
21. Marret S, Delpech B, Delpech A, et al. Expression and effects of hyaluronan and of the hyaluronan-binding protein hyaluronectin in newborn rat brain glial cell cultures. *J Neurochem* 1994;62:1285–1295.
22. Lee HG, Cowman MK. An agarose gel electrophoretic method for analysis of hyaluronan molecular weight distribution. *Anal Biochem* 1994;219:278–287.
23. Botzki A, Rigden DJ, Braun S, et al. L-Ascorbic acid 6-hexadecanoate, a potent hyaluronidase inhibitor. X-ray structure and molecular modeling of enzyme-inhibitor complexes. *J Biol Chem* 2004;279:45990–45997.
24. Baggenstoss BA, Weigel PH. Size exclusion chromatography-multiple laser light scattering analysis of hyaluronan size distributions made by membrane-bound hyaluronan synthase. *Anal Biochem* 2006;352:243–251.
25. Dissen GA, Lomniczi A, Neff TL, et al. In vivo manipulation of gene expression in non-human primates using lentiviral vectors as delivery vehicles. *Methods* 2009;49:70–77.
26. Atmuri V, Martin DC, Hemming R, et al. Hyaluronidase 3 (HYAL3) knockout mice do not display evidence of hyaluronan accumulation. *Matrix Biol* 2008;27:653–660.
27. Zhang SC, Lundberg C, Lipsitz D, et al. Generation of oligodendroglial progenitors from neural stem cells. *J Neurocytol* 1998;27:475–489.
28. Meyer MF, Kreil G, Aschauer H. The soluble hyaluronidase from bull testes is a fragment of the membrane-bound PH-20 enzyme. *FEBS Lett* 1997;413:385–388.
29. Phelps B, Myles DG. The guinea pig sperm plasma membrane protein, PH-20, reaches the surface via two transport pathways and becomes localized to a domain after an initial uniform distribution. *Dev Biol* 1987;123:63–72.
30. Jones MH, Davey PM, Aplin H, Affara NA. Expression analysis, genomic structure, and mapping to 7q31 of the human sperm adhesion molecule gene SPAM1. *Genomics* 1995;29:796–800.
31. Jones R, Ma A, Hou ST, et al. Testicular biosynthesis and epididymal endoproteolytic processing of rat sperm surface antigen 2B1. *J Cell Sci* 1996;109(pt 10):2561–2570.
32. Zheng Y, Martin-DeLeon PA. The mouse Spam1 gene: RNA expression pattern and lower steady-state level associated with the Rb(6.16) translocation. *Mol Reprod Dev* 1997;46:252–257.
33. Wang H, Zhan Y, Xu L, et al. Use of suppression subtractive hybridization for differential gene expression in stroke: discovery of CD44 gene expression and localization in permanent focal stroke in rats. *Stroke* 2011;32:1020–1027.
34. Jenkins HG, Bachelard HS. Developmental and age-related changes in rat brain glycosaminoglycans. *J Neurochem* 1988;51:1634–1640.
35. Jenkins HG, Bachelard HS. Glycosaminoglycans in cortical autopsy samples from Alzheimer brain. *J Neurochem* 1988;51:1641–1645.
36. Suzuki K, Katzman R, Korey SR. Chemical studies on Alzheimer's disease. *J Neuropathol Exp Neurol* 1965;24:211–224.
37. Contreras EG, Gaete M, Sánchez N, et al. Early requirement of hyaluronan for tail regeneration in *Xenopus* tadpoles. *Development* 2009;136:2987–2996.
38. Fancy SP, Baranzini SE, Zhao C, et al. Dysregulation of the Wnt pathway inhibits timely myelination and remyelination in the mammalian CNS. *Genes Dev* 2009;23:1571–1585.
39. Feigenson K, Reid M, See J, et al. Wnt signaling is sufficient to perturb oligodendrocyte maturation. *Mol Cell Neurosci* 2009;42:255–265.
40. Azim K, Butt AM. GSK3 β negatively regulates oligodendrocyte differentiation and myelination in vivo. *Glia* 2011;59:540–553.
41. Tawk M, Makoukji J, Belle M, et al. Wnt/ β -catenin signaling is an essential and direct driver of myelin gene expression and myelinogenesis. *J Neurosci* 2011;31:3729–3742.
42. Termeer C, Benedix F, Sleeman J, et al. Oligosaccharides of hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med* 2002;195:99–111.
43. Taylor KR, Trowbridge JM, Rudisill JA, et al. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem* 2004;279:17079–17084.
44. Jiang D, Liang J, Fan Y, et al. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat Med* 2005;11:1173–1179.
45. Scheibner KA, Lutz MA, Boodoo S, et al. Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J Immunol* 2006;177:1272–1281.
46. Shimada M, Yanai Y, Okazaki T, et al. Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. *Development* 2008;135:2001–2011.
47. Kim JS, Yeo S, Shin DG, et al. Glycogen synthase kinase 3 β and β -catenin pathway is involved in toll-like receptor 4-mediated NADPH oxidase 1 expression in macrophages. *FEBS J* 2010;277:2830–2837.
48. Zhang P, Katz J, Michalek SM. Glycogen synthase kinase-3 β (GSK3 β) inhibition suppresses the inflammatory response to Francisella infection and protects against tularemia in mice. *Mol Immunol* 2009;46:677–687.
49. Wang Y, Cheng X, He Q, et al. Astrocytes from the contused spinal cord inhibit oligodendrocyte differentiation of adult oligodendrocyte precursor cells by increasing the expression of bone morphogenetic proteins. *J Neurosci* 2011;31:6053–6058.
50. Mi S, Hu B, Hahm K, et al. LINGO-1 antagonist promotes spinal cord remyelination and axonal integrity in MOG-induced experimental autoimmune encephalomyelitis. *Nat Med* 2007;13:1228–1233.
51. Stern R, Jedrzejewski MJ. Hyaluronidases: their genomics, structures, and mechanisms of action. *Chem Rev* 2006;106:818–839.
52. Martin DC, Atmuri V, Hemming RJ, et al. A mouse model of human mucopolysaccharidosis IX exhibits osteoarthritis. *Hum Mol Genet* 2008;17:1904–1915.
53. Triggs-Raine B, Salo TJ, Zhang H, et al. Mutations in HYAL1, a member of a tandemly distributed multigene family encoding disparate hyaluronidase activities, cause a newly described lysosomal disorder, mucopolysaccharidosis IX. *Proc Natl Acad Sci U S A* 1999;96:6296–6300.
54. Jadin L, Wu X, Ding H, et al. Skeletal and hematological anomalies in HYAL2-deficient mice: a second type of mucopolysaccharidosis IX? *FASEB J* 2008;22:4316–4326.
55. Baba D, Kashiwabara S, Honda A, et al. Mouse sperm lacking cell surface hyaluronidase PH-20 can pass through the layer of cumulus cells and fertilize the egg. *J Biol Chem* 2002;277:30310–30314.