

著者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Zou K, Liu J, Watanabe A, Hiraga S, Liu S, Tanabe C, Maeda T, Terayama Y, Takahashi S, <u>Michikawa M</u> , Komano H	Aβ43 is the earliest depositing Aβ species in APP transgenic mouse brain and is converted to Aβ41 by two active domains of ACE	<i>Am. J. Pathol.</i>		In press	2013
Hosono-Fukao T, Ohtake-Niimi S, Hoshino H, Akatsu H, Hossain M, Nishitsuji K, van Kuppevelt, Kimata K, <u>Michikawa M</u> , Wyss-Coray T, Uchimura K.	Heparan Sulfate Subdomains that are Degraded by Sulf Accumulate in Cerebral Amyloid β Plaques of Alzheimer's Disease : Evidence from Mouse Models and Patients	<i>Am. J. Pathol.</i>	180	2056-2067	2012
Yasuno F, Tanimukai S, Sasaki M, Hidaka S, Ikejima C, Yamashita F, Kodama C, Mizukami K, <u>Michikawa M</u> , Asada T	Association between cognitive function and plasma lipids of the elderly after controlling for apolipoprotein E genotype.	<i>Am. J. Geriat. Psychiat</i>	20	574-583	2012

著者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kakiya, N., Saito, T., Nilsson, P., Matsuba, Y., Tsubuki, S., Takei, N., Nawa, H., <u>Saido T.C.</u>	Cell-surface expression of the major A β degrading enzyme, neprilysin, depends on phosphorylation by MEK and dephosphorylation by protein phosphatase 1a.	<i>J. Biol. Chem.</i> ,	287	29362-29 372	2012
Abramowski, D., Rabe, S., Upadhaya, A.R., Reichwald, J., Danner, S., Staab, D., Capetillo-Zarate, E., Yamaguchi, H., <u>Saido, T.C.</u> , Wiederhold, K.H., Thal, D.R., Staufenbiel, M.	Transgenic expression of intra-neuronal A β 42 but not A β 40 leads to cellular A β lesions, degeneration, and functional impairment without typical Alzheimer's disease pathology.	<i>J. Neurosci.</i>	32	1273-128 3	2012
Schlenzig, D., Rönicke, R., Cynis, H., Ludwig, H.H., Scheel, E., Reymann, K., <u>Saido, T.</u> , Hause, G., Schilling, S., Demuth, H.U.	N-terminal Pyroglutamate (pGlu) formation of A β 38 and A β 40 Enforces Oligomer Formation and Potency to Disrupt Hippocampal LTP.	<i>J. Neurochem.</i>	121	774-784	2012
Iwata, N., Sekiguchi, M., Hattori, Y., Takahashi, A., Asai, M., Ji, B., Higuchi, M., Staufenbiel, M., Muramatsu, S., <u>Saido T.C.</u>	Global brain delivery of neprilysin gene by intravascular administration of AAV vector in mice.	<i>Sci. Rep.</i>		in press	2013

2012年成果表

著者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Takeo K, Watanabe N, Tomita T*, Iwatsubo T	Contribution of γ -secretase cofactors to the formation of catalytic pore of presenilin 1.	<i>J Biol Chem</i>	287	25834-25843	2012
Suzuki K, Hayashi Y, Nakahara S, Kumazaki H, Prox J, Horiuchi K, Zeng M, Tanimura S, Nishiyama Y, Osawa S, Sehara-Fujisawa A, Saftig P, Yokoshima S, Fukuyama T, Matsuki N, Koyama R, Tomita T*, Iwatsubo T	Activity-dependent Cleavage of Neuroigin 1.	<i>Neuron</i>	76	410-422	2012
Takagi-Niido me S, Osawa S, Tomita T*, Iwatsubo T	Inhibition of γ -secretase activity by a monoclonal antibody against the extracellular hydrophilic loop of presenilin 1.	<i>Biochemistry</i>	52	61-69	2013
Imamura Y, Umezawa N, Osawa S, Iwatsubo T, Kato N, Tomita T*, Higuchi T	Effect of helical conformation and side-chain structure on γ -secretase inhibition by β -peptide foldamers: Insight into substrate recognition.	<i>J Med Chem</i>			In press

2012 年度成果表

著者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Masuda-Suzukake M, Nonaka T, Hosokawa M, Oikawa T, Arai T, Akiyama H, Mann D, Hasegawa M,	Prion-like spreading of pathological alpha-synuclein in brain.	Brain		in press	2013
Ogaki K, Li Y, Takanashi M, Ishikawa KI, Kobayashi T, Nonaka T, Hasegawa M, Kishi M, Yoshino H, Funayama M, Tsukamoto T, Shioya, Y Kokochi M, Imai H, Sasaki R, Kokubo Y, Kuzuhara S, Motoi Y, Tomiyama H, Hattori N.	Analyses of the MAPT, PGRN, and C9orf72 mutations in Japanese patients with FTLT, PSP, and CBS.	Parkinsonism & Related Disorders	19	15-20.	2013
Hosokawa M, Arai T, Masuda-Suzukake M, Nonaka T, Yamashita M, Akiyama H, Hasegawa M	Methylene blue reduced abnormal tau accumulation in P301L tau transgenic mice.	PLoS One	7	e52389	2012
Tsuji H, Arai T, Kametani F, Nonaka T, Yamashita M, Suzukake M, Hosokawa M, Yoshida M, Hatsuta H, Takao M, Saito Y, Murayama M, Akiyama H, Hasegawa M, Mann DMA, Tamaoka A.	Molecular analysis and biochemical classification of TDP-43 proteinopathy.	Brain	135	3380-3391	2012
Egawa N, Kitaoka S, Tsukita K, Naitoh M, Takahashi K, Yamamoto T, Adachi F, Kondo T, Okita K, Asaka I, Takashi Aoi T, Watanabe A, Yamada Y, Morizane A, Takahashi J, Ayaki T, Ito H, Yoshikawa K, Yamawaki S, Suzuki S, Watanabe D, Hioki H, Kaneko T, Makioka K, Okamoto K, Takuma H, Tamaoka A, Hasegawa K, Nonaka T, Hasegawa M, Kawata A, Yoshida M, Nakahata T, Takahashi R, Marchetto MCN, Gage FH, Yamanaka S, Inoue H. A (2012).	Drug-Screening Platform for ALS Using Patient-Specific Induced Pluripotent Stem Cells.	Sci Transl Med	4	145ra104	2012
Parker SJ, Meyerowitz J, James JL, Liddell JR, Nonaka T, Hasegawa M, Kanninen KM, Lim SC, Paterson BM, Donnelly PS, Crouch PJ, White AR.	Inhibition of TDP-43 accumulation by bis(thiosemicarbazono)-copper complexes.	PLoS One	7	e42277.	2012
Kokubo Y, Taniguchi A, Hasegawa M, Hayakawa Y, Morimoto S, Yoneda M, Hirokawa Y, Shiraishi T, Saito Y, Murayama S, Kuzuhara S.	α -Synuclein Pathology in the Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex in the Kii Peninsula, Japan.	J Neuropathol Exp Neurol.	71	625-30.	2012

Wang Y, Shi M, Chung KA, Zabetian CP, Leverenz JB, Berg D, Surljies K, Trojanowski JQ, Lee VMY, Siderowf AD, Hurtig H, Litvan I, Schiess MC, Peskind E, Masuda M, Hasegawa M, Lin X, Pan C, Galasko D, Goldstein DS, Jensen PH, Yang H, Cain KC, Zhang J	Phosphorylated a-Synuclein in Parkinson's Disease.	Sci Transl Med.	4	121ra20	2012
Shahpasand K, Uemura I, Saito T, Asano T, Hata K, Shibata K, Toyoshima Y, Hasegawa M, and Hisanaga S.	Regulation of mitochondrial transport and inter-microtubule spacing by Tau phosphorylation at the sites hyperphosphorylated in Alzheimer's disease.	J Neurosci.	32	2430-2441	2012
Takahashi M, China Y, Nonaka T, Hasegawa M, Watanabe N, Arai T.	Prolonged nitric oxide treatment induces tau aggregation in SH-SY5Y cells,	Neurosci Lett	510	48- 52	2012

IV.研究成果の刊行物・別刷

Matrix Pathobiology

Heparan Sulfate Subdomains that are Degraded by Sulf Accumulate in Cerebral Amyloid β Plaques of Alzheimer's Disease

Evidence from Mouse Models and Patients

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Alzheimer's disease (AD) is characterized by extracellular cerebral accumulation of amyloid β peptide (A β). Heparan sulfate (HS) is a glycosaminoglycan that is abundant in the extracellular space. The state of sulfation within the HS chain influences its ability to interact with a variety of proteins. Highly sulfated domains within HS are crucial for A β aggregation *in vitro*. Here, we investigated the expression of the sulfated domains and HS disaccharide composition in the brains of Tg2576, J20, and T41 transgenic AD mouse models, and patients with AD. RB4CD12, a phage display antibody, recognizes highly sulfated domains of HS. The RB4CD12 epitope is abundant in the basement membrane of brain vessels under physiological conditions. In the cortex and hippocampus of the mice and patients with AD, RB4CD12 strongly stained both diffuse and neuritic amyloid plaques. Interestingly, RB4CD12 also stained the intracellular granules of certain hippocampal neurons in AD brains. Disaccharide compositions in vessel-enriched and nonvasculature fractions

of Tg2576 mice and AD patients were found to be comparable to those of non-transgenic and non-demented controls, respectively. The RB4CD12 epitope in amyloid plaques was substantially degraded *ex vivo* by Sulf-1 and Sulf-2, extracellular HS endosulfatases. These results indicate that formation of highly sulfated HS domains may be upregulated in conjunction with AD pathogenesis, and that these domains can be enzymatically remodeled in AD brains. (Am J Pathol 2012, 180: 2056–2067; DOI: 10.1016/j.ajpath.2012.01.015)

Heparan sulfate (HS) is a linear polysaccharide that exists in large quantities in the extracellular space. One or more HS chains are covalently bound to a core protein comprising heparan sulfate proteoglycan (HSPG).^{1,2} HS chains and heparins, structural analogues of HS chains, are a family of glycosaminoglycans consisting of repeating disaccharide units of glucuronic/iduronic acid and glucosamine. Modification with sulfation as well as elongation of these disaccharides is enzymatic,³ bestowing on the chains structural diversity.^{4–6} HS contains highly

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sulfated domains and partially sulfated or non-sulfated domains, which are transitional.³ Highly sulfated domains are formed by consecutive clusters of sulfated disaccharides. It has been shown that a trisulfated disaccharide structure [-iduronic acid(2S)-Glucosamine(NS,6S)-] occurs within highly sulfated domains. RB4CD12, a phage display anti-HS antibody, has been shown to recognize trisulfated disaccharide-containing HS subdomains⁷⁻⁹ Trisulfated disaccharides are considered to be key elements in molecular interactions between HS/heparin and many ligands, including growth factors and morphogens.^{1,10} Trisulfated disaccharides, as well as the RB4CD12 epitope, are degraded by extracellular sulfatases, Sulf-1, and Sulf-2.^{8,11,12} In the brain, we have shown that the RB4CD12 HS domains are abundantly present in the vasculature⁹ and that these domains can be degraded by the Sulfs *ex vivo*.⁸ However, the roles of the RB4CD12 HS domains in pathological and physiological processes in brain vasculature are not known.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. One of the pathological hallmarks of AD is the presence of extracellular amyloid plaques in brain areas that are responsible for cognition and memory functions. The predominant composition of amyloid plaques is fibrils made of amyloid β peptide ($A\beta$). A great deal of biochemical and genetic evidence has indicated that aggregation and accumulation of $A\beta$ in toxic forms within the extracellular space play a central role in AD pathogenesis. One of the authors previously reported that certain structures of HS chains exist in amyloid plaques of AD brains,¹³ and that structural variation of HSPG correlates with amyloid plaque formation in the brains of AD patients.¹⁴ HSPG is also known to facilitate cerebral amyloid deposition induced exogenously in a rat model *in vivo*.¹⁵ Functional roles of HS and HSPG in AD pathology are proposed to be acceleration of $A\beta$ fibril formation and protection of the fibril against microglial phagocytosis.¹⁶ It was reported that the aggregation state of $A\beta$ requires its binding properties to heparin.¹⁷ Pathological correlations between the RB4CD12 HS domains, which are rich in heparin and AD have not been established. Here we present evidence that the RB4CD12 HS domains are accumulated in cerebral amyloid plaques of transgenic AD mouse models and patients with AD, and that these HS epitopes can be degraded by Sulf-1 and Sulf-2 *ex vivo*.

Materials and Methods

Materials

The RB4CD12 phage display-derived anti-heparan sulfate antibody was produced in a vesicular stomatitis virus (VSV)-tag version and purified as previously described.⁷ Alternative nomenclature of RB4CD12 is HS3A8. The following materials were commercially obtained from the sources indicated. Heparinases (I, II and III), polyclonal rabbit anti-laminin antibody (Ab), horseradish peroxidase-conjugated monoclonal anti-VSV Ab, and Cy3-conjugated monoclonal anti-VSV Ab were from Sigma (St.

Louis, MO); biotinylated monoclonal anti-amyloid β (N-terminus) Ab (82E1) was from IBL (Gunma, Japan); polyclonal rabbit anti-VSV Ab was from Bethyl Laboratories (Montgomery, TX); Cy2-conjugated goat anti-mouse IgG (H+L), Cy2-conjugated goat anti-rabbit IgG (H+L), Cy2-conjugated goat anti-rat IgG (H+L) Abs, and Cy2-conjugated streptavidin were from Jackson ImmunoResearch Laboratories (West Grove, PA); rabbit anti-Iba1 Ab was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); rabbit anti-gial fibrillary acidic protein and monoclonal anti-phospho-PHF-tau pThr231 (AT180) Abs were from Thermo Scientific (Rockford, IL); goat anti-mouse syndecan-3 Ab was from R&D Systems, Inc (Minneapolis, MN); rabbit anti-glypican-1 (M-95) Ab was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA); polyclonal goat anti-rabbit IgG Nanogold, ϕ 1.4 nm, was from Nanoprobe (Yaphank, NY); and horseradish peroxidase-conjugated goat anti-rabbit IgG was from Cell Signaling Technology, Inc. (Beverly, MA).

Animals

C57BL/6 mice were from Japan SLC Inc. (Hamamatsu, Japan). Heterozygotic transgenic mice that expressed the human amyloid precursor protein bearing the Swedish (K670N, M671L) mutation (Tg2576 strain),¹⁸ the Swedish and Indiana (V717F) mutations (J20 strain),¹⁹ or the Swedish and London (V717I) mutations (T41 strain)²⁰ were maintained in barrier facilities. Tg2576 mice were purchased from Taconic Farms, Inc., Hudson, NY. J20 mice were from the Jackson Laboratory (Bar Harbor, ME). The National Center of Geriatrics and Gerontology Institutional Animal Care and Use Committee approved the animal studies.

Human Postmortem Brain Tissues

Patients with sporadic AD received a pathological diagnosis according to the criteria of the Consortium to Establish a Registry for Alzheimer's Disease and the Braak stage. Non-demented controls were elderly patients who were age-matched and without significant neurological disorders. Patients were also cognitively evaluated by neuropsychological tests using the Mini-Mental State Examination and Hasegawa's dementia scale, which is commonly used in Japan. Entorhinal cortex and hippocampus postmortem tissue samples from neurologically unimpaired subjects (non-demented controls [NDCs]) and from subjects with AD were obtained under Committees on Human Research approval of National Center for Geriatrics and Gerontology and Choku Medical Institute of Fukushima Hospital. Diagnosis of AD was confirmed by pathological and clinical criteria (Table 1). The incidence of vascular risk factors (eg, atherosclerosis, myocardial infarction, and so forth), the sex ratio, age, and the postmortem interval were comparable between NDC and AD (Table 1). Tissue was cut and frozen or fixed with formalin, and then embedded with paraffin. Frozen tissues were subjected to structural analysis of HS. The embedded tissues were cut using a microtome.

Table 1. Clinical and Neuropathological Characteristics of Alzheimer's Disease and Non-Demented Control Donor Patients used in the Disaccharide Composition Analysis of Heparan Sulfate

Patient number	Age (years)	Sex	Stage of amyloid deposits (0, A, B, C)*	NFT stage (I–VI)	Cerebral amyloid angiopathy	Vascular risk factors	PMI (hr)
Alzheimer's disease patients							
0508	94	F	C	V	+	CI	43
0512	83	F	C	VI	+	ATH	2
0604	91	F	C	V	–	CI	8
0805	93	F	C	VI	+	CI	27
0810	80	M	C	V	–	CI	15
0811	81	M	C	VI	–	–	8
0814	91	M	C	V	+	–	5
0824	87	F	B	VI	–	–	9
Age-matched non-demented controls							
0707	95	F	A	II	–	MI	4
0710	83	F	A	II	–	CH/CI	24
0601	90	F	B	II	–	MI	4
0802	93	F	A	III	–	CH/CI	20
0704	84	M	B	II	–	CI	3
0807	82	M	0	I	–	CH	8
0908	91	M	A	II	–	–	NA
0903	87	F	0	II	–	CI	7

*0 = none, A = rare or a few, B = mild or moderate, C = numerous or marked.

ATH, atherosclerosis; CH, cerebral hemorrhage; CI, cerebral infarction; F, female; M, male; MI, myocardial infarction; NA, not applicable; NFT, neurofibrillary tangle; PMI, postmortem interval.

Fractionation of Brain Samples

A snap-frozen mouse cortex (~25 mg) was placed in a tube containing 600 μ L (30 volume of the tissue weight) of ice-cold Tris-buffered saline (TBS) (20 mmol/L Tris and 137 mmol/L NaCl, pH 7.6) and protease inhibitors (complete protease inhibitor cocktail; Roche Diagnostics, Mannheim, Germany). The tube was placed in a water bath of the Bioruptor ultrasonic vibration (CosmoBio, Tokyo). The tissue was fragmented by sonicating the tube for 15 seconds with the maximum ultrasonic wave output power 4 to 5 times until solid materials in the tube became invisible. The material was ultracentrifuged at 100,000 $\times g$ for 20 minutes at 4°C. The supernatant was collected and stored frozen as TBS or "TBS soluble fraction." The resulting precipitate was suspended in 600 μ L (the same volume as previously described) of TBS containing 1% SDS. The suspension was centrifuged at 12,000 rpm for 20 minutes at room temperature. The resulting supernatant was collected and stored frozen as TBS or "TBS-insoluble/1% SDS-soluble fraction." The protein concentrations of both fractions were measured with a BCA Protein Assay Reagent Kit (Thermo Scientific). Brain cortices were dissected out from 3 Non-Tg or 3 Tg2576 18-month-old mice and then snap frozen. Brain samples were put together, placed on a glass Petri dish, and minced with a blade. The tissues were transferred into a tube containing 1 mL of ice-cold TBS. The tissues were homogenized with a Dounce homogenizer. The homogenate was filtered with a 100- μ m nylon mesh. The filtered materials on the mesh were collected and then subjected to the structural analysis described as follows ("vessel-enriched fractions"). Materials filtered through the 100- μ m nylon mesh were collected and then analyzed ("non-vasculature fractions"). Methylene blue staining and bright field microscopy confirmed cerebral blood vessels on the filters.

Immunohistochemistry

Fresh mouse brains were embedded in O.C.T. compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. The brains were stored at –80°C until analysis. Cryostat-cut sections (10- μ m thick) were prepared on MAS-coated glass slides (Matsunami, Osaka, Japan), fixed in ice-cold acetone for 15 minutes, and then air-dried for 30 minutes. Sections were incubated with blocking solution (3% bovine serum albumin in PBS) for 15 minutes at RT. Sections were washed twice with PBS and then incubated with a mixture of RB4CD12 (1:100 dilution), rabbit anti-laminin antibody (1:100 dilution, Sigma), and biotinylated 82E1 (1:50 dilution) overnight at 4°C. Then, primary antibodies were detected with Cy3-conjugated monoclonal anti-VSV-G (4 μ g/mL), Cy2-conjugated polyclonal goat anti-rabbit IgG (3 μ g/mL), and aminomethylcoumarin acetate-conjugated streptavidin (6.8 μ g/mL, Jackson ImmunoResearch, West Grove, PA). Sections were mounted in FluorSave Reagent (Merck, Darmstadt, Germany). Digital images were captured by fluorescent microscopy (model BX50, Olympus, Tokyo, Japan) at the same setting for each antibody. The fluorescently stained area was quantitatively determined using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). To determine the effects of the Sulfs and heparinases, 3% bovine serum albumin-blocked sections were pre-treated with 100 μ L of a reaction mixture containing 5 μ mol HEPES, pH 7.5, 1 μ mol MgCl₂, and enzymes at 37°C overnight. Recombinant human Sulf-1 (0.4 μ g) and human Sulf-2 (0.4 μ g) were prepared from conditioned medium of transfected HEK293 cells and used as previously described.⁸ For pretreatment with heparitinases, a mixture of 1 mU heparinase I, 0.25 mU heparinase II, and 0.1 mU heparinase III were added to the

reaction mixture. Cy2-conjugated streptavidin was used to detect bound 82E1. Human brain sections (4- μ m thickness) were obtained from paraffin-embedded tissue blocks. After deparaffinization and rehydration, endogenous peroxidase activity was quenched with 3% H₂O₂ (Sigma). Sections were subjected to heat-induced epitope retrieval followed by IgG blocking using M.O.M. kit (Vector Laboratories Inc., Burlingame, CA). Sections were incubated with RB4CD12 (1:100 dilution) overnight at 4°C. Bound antibody was detected with horseradish peroxidase-conjugated mouse anti-VSV followed by visualization with diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride) supplied with the EnVision reagent (Dako Japan, Tokyo, Japan).

Immunoelectron Microscopy

Cryostat-cut sections from 17-month-old Tg2576 mouse brains were prepared on MAS-coated glass slides, fixed in 4% paraformaldehyde for 5 minutes, and then washed with PBS for 1 hour. Sections were incubated with 3% bovine serum albumin for 30 minutes at RT. Diluted RB4CD12 antibody (1:40) was then applied overnight. After washing, diluted rabbit anti-VSV secondary antibody (7.2 μ g/mL) was applied for 1 hour. After several washes, diluted goat anti-rabbit IgG antibody coupled with 1.4-nm-diameter tertiary gold particles (1:40) was applied for 30 minutes. The samples were then washed and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 3 hours, followed by enlargement of the gold particles with an HQ-Silver Enhancement Kit (Nanoprobes). The specimens were examined in a Hitachi H-7600 transmission electron microscope (Hitachi Koki, Tokyo, Japan).

Immunoblots

The proteins (40 μ g per lane) were separated by NuPAGE 3% to 8% polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, CA), and blotted onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked with 5% skim milk/PBS 0.1% Tween for 1 hour at room temperature and then incubated overnight with RB4CD12 antibody (1:500) in TBS 0.1% Tween at 4°C. The membrane was washed and incubated with horseradish peroxidase-conjugated mouse anti-VSV (1:2000) for 1 hour at RT. Bound antibodies were visualized with SuperSignal West Dura Chemiluminescent reagent (Thermo Scientific). Signals were visualized and quantified using a LAS-3000 mini luminescent image analyzer (Fujifilm, Tokyo, Japan).

Preparation and Structural Analysis of HS

There were 100 mg of frozen brain tissues or the cortical vessel residue that remained on filters previously described, which was suspended in 2 mL of 0.2N NaOH and incubated overnight at RT. The samples were neutralized with 4 N HCl and then treated with DNase I and RNase A (0.04 mg/mL each) (Roche Diagnostics) in 50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L MgCl₂ for 3 hours at 37°C. Subsequently, the samples were treated with acti-

nase E (0.08 mg/mL) (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) overnight at 37°C. The supernatant was collected by centrifugation at 5000 \times *g* at 4°C for 10 minutes after heat inactivation of the enzyme and then mixed with the same volume of 50 mmol/L Tris-HCl, pH 7.2. The HS was purified by DEAE-Sepharose column chromatography.⁹ The disaccharide compositions of the HS were determined by reversed-phase ion-pair chromatography with postcolumn fluorescent labeling.

Quantitative Real Time-PCR for Expression of Genes Related to HS Synthesis

Total RNA was extracted from frozen mouse cortices using TRIzol Reagent (Invitrogen). Total RNA (4 μ g) was used for reverse transcription reaction in 100 μ L of buffer with random hexamers, using Superscript II Reverse Transcriptase (Invitrogen). PCR was conducted in duplicate with 20- μ L reaction volumes of SYBR Premix Ex TaqII (Takara Bio Inc. Shiga, Japan), 0.2 μ mol/L of each primer and 2 μ L of the cDNA reaction mixture. PCR was performed using the following parameters: 95°C, 10 seconds, 1 cycle; 95°C, 5 seconds; and 60°C, 30 seconds, 40 cycles. Analysis was performed using sequence detection software supplied with Thermal Cycler Dice Real Time System TP800 (Takara Bio Inc.). mRNA levels of each gene were normalized by comparison to β -actin mRNA levels. Conclusions are drawn from duplicate PCR reactions at least two independent reverse transcription reactions. Primer sequences used in this study are as indicated for *Ndst1*, 5'-GCAGATGGCCCTGAACAA-GAA-3' and 5'-GCACGTGCACAGGGTACACA-3'; for N-deacetylase/N-sulfotransferase 2 (*Ndst2*), 5'-TCATCCAG-AAGTTCTGGGTATCAC-3' and 5'-AGACAGCGAGTC-TTACCACCTTCAA-3'; for *Ndst3*, 5'-TCTGGTGTCAGCT-GCTGGAAG-3' and 5'-CACGTTGTGGTCGCGGTAGTAG-3'; for *Ndst4*, 5'-TTGTTCCAAAGCCAAGATCATTAC-3' and 5'-TCAGGGCAGCTGGATCTTCA-3'; for *Hs6st1*, 5'-CT-GACTGGACCGAACTCACAA-3' and 5'-TCTCGCAGC-AGGGTGATGTAGTAG-3'; for *Hs6st2*, 5'-AAACTTCAACT-CAGGCGCCAAC-3' and 5'-CTCCATTCCTCAAGTACCGT-GACA-3'; for *Hs6st3*, 5'-GACTGGACCGAGCTCACAA-3' and 5'-CATGCTTCCATTGCTCAGGTA-3'; for *Hs2st1*, 5'-GCAAGCACCTCGTTCACAA-3' and 5'-CATCTCGTTC-CAGGTGGTTATGTTTC-3'; for *Sulf1*, 5'-CCACATGGAGTT-CACCAACGTC-3' and 5'-TAGCCGTGGTCCGAGTGTA-3'; for *Sulf2*, 5'-GAGTACCAGACAGCATGCCAACA-3' and 5'-TTGGGCACCAGTTGGAGA-3'; and for *Actb*, 5'-CATCCG-TAAAGACCTCTATGCCAAC-3' and 5'-ATGGAGCCAC-CGATCCACA-3'.

Statistical Analysis

All data are presented as means \pm SD unless noted otherwise. The values were analyzed by unpaired Student's *t*-test using Prism software (GraphPad Software, La Jolla, CA). *P* values less than 0.05 were considered to be statistically significant.

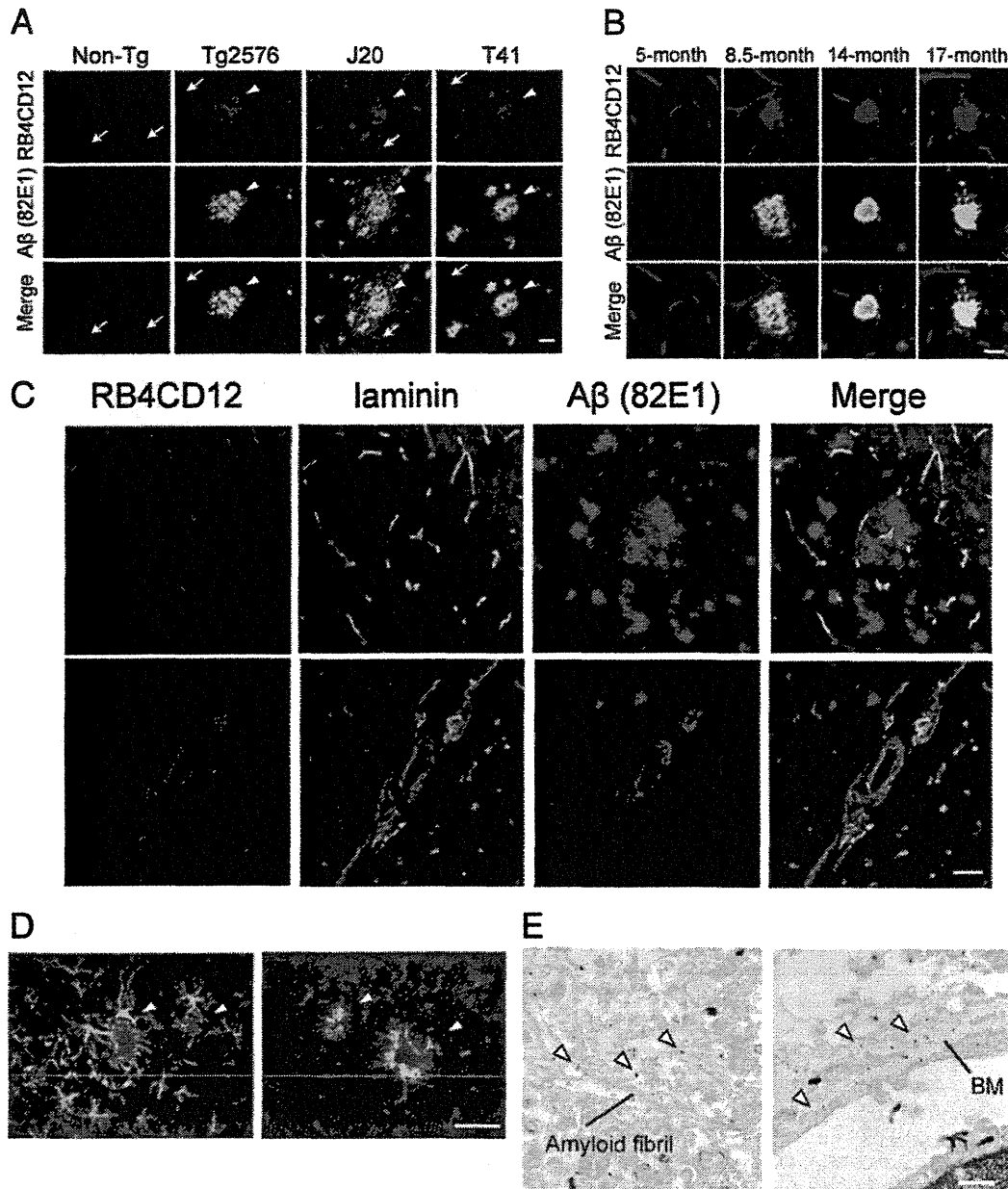


Figure 1. RB4CD12 anti-heparan sulfate epitope colocalizes with amyloid β plaques in the brain of Tg2576, J20, and T41 mice. **A:** Cryostat-cut brain sections of 18-month-old non-Tg and Tg2576, 23-month-old J20, and 12-month-old T41 mice were stained with RB4CD12 anti-HS antibody (red) and 82E1 anti-amyloid β (A β) antibody (green). Staining signals in vessels (**arrows**) and amyloid plaques (**arrowheads**) are shown. **B:** Expression of the RB4CD12 epitope and A β in aging Tg2576 brain. **C:** Expression of the RB4CD12 epitope, A β and laminin, a marker for vessels in the 18-month-old Tg2576 brain. **D:** Co-staining of RB4CD12 anti-HS antibody (red) with cell type-specific antibodies against glial fibrillary acidic protein astrocytes (**left**) or Iba1 microglia (**right**) (green). Co-stained areas are shown in yellow. **E:** Immunoelectron microscopy for the RB4CD12 epitope in amyloid fibrils in the brain of 18-month-old Tg2576 mouse. **Left panel** shows RB4CD12 signals in amyloid fibrils indicated by **arrowheads**. **Right panel** shows RB4CD12 signals in the basement membrane of vessels. BM, basement membrane. Scale bars (in **A**, **C**, and **D**): 50 μ m. Scale bars (in **B** and **E**): 20 μ m and 500 nm, respectively.

Results

Immunoreactivity of RB4CD12 Anti-Heparan Sulfate is Colocalized with A β Plaques in Brains of Transgenic Mouse Models of AD

RB4CD12 scFv antibody recognizes trisulfated disaccharide-containing highly sulfated domains within HS.^{8,21} The RB4CD12 epitope has been shown to be present

abundantly in the vasculature of the brain in mice.⁹ We first analyzed expression of the RB4CD12 epitope in the brain of transgenic mouse models of AD. Tg2576 mice express mutated human amyloid precursor protein in the brain and show numerous A β plaques in the cortex and hippocampus.¹⁸ The localization of the RB4CD12 highly sulfated domains in A β plaques was observed in an 18-month-old Tg2576 hippocampus (Figure 1A). The RB4CD12 epitope was immunolocalized in both diffuse

and neuritic amyloid plaques in the brain of Tg2576 (Figure 1, A–C). RB4CD12 also detected brain microvessels in Tg2576 mice. No specific staining was observed when RB4CD12 was substituted with MPB49, a non-HS scFv antibody (not shown). We also tested aged J20 and T41, other mouse models of AD. With respect to expression levels of A β peptides, A β 42 is dominant in J20 and T41 mouse brains, whereas A β 40 is dominant in Tg2576 mouse. We examined brain sections of these model mice immunohistochemically. The RB4CD12 highly sulfated domains were colocalized with A β plaques in the hippocampus of 23-month-old J20 and 12-month-old T41 mice (Figure 1A). To analyze age-dependent accumulation of the RB4CD12 epitope in A β plaques, we collected Tg2576 brains from 5-, 8.5-, 14- and 17-month-old mice. A β plaques were observed in 8.5-, 14- and 17-month-old Tg2576 brains. Cerebral A β deposition increases with age. RB4CD12 stained A β plaques at these ages (Figure 1B). Next, we investigated vasculature and non-vasculature RB4CD12 epitopes in aged Tg2576 brain by costaining with antibodies against A β and laminin, a marker of vascular basement membranes. Immunoreactivity of RB4CD12 in vascular structure was colocalized with anti-laminin staining signals (Figure 1C). RB4CD12 staining signals that were not associated with signals of anti-laminin antibody predominantly colocalized with anti-A β staining signals in the cortex of Tg2576 mice (Figure 1C, upper panels). The RB4CD12 epitope was also observed in the vessel walls of A β -positive leptomeningeal vessels (Figure 1C, lower panels). Staining patterns of RB4CD12 were different from the immunoreactivity of glial fibrillary acidic protein, an astrocyte marker, and Iba-1, a microglia marker (Figure 1D). Immunoelectron microscopy confirmed the localization of RB4CD12 epitope within amyloid fibrils and the basement membrane (Figure 1E). The RB4CD12 immunoreactive area that is not colocalized with anti-laminin staining signals was determined by fluorescence microscopy and quantified with computer-aided image analysis. In Tg2576 cortex and hippocampus, RB4CD12-positive but laminin-negative area was increased to fourfold to fivefold of that in non-Tg (Figure 2). In contrast, no change was observed in the cerebellum where no A β plaques were observed (Figure 2). We noted that laminin-positive vessels had attenuated diameters and a more ragged profile in Tg2576 cortex and hippocampus (Figure 2).

The RB4CD12 Epitope Is Immunolocalized in Amyloid Plaques in Postmortem Brains of Alzheimer's Disease Patients

We tested RB4CD12 antibody for staining of brains from NDC individuals and AD patients (Table 1). In NDCs, vessel-staining signals were predominantly observed (Figure 3A). In AD entorhinal cortex, amyloid deposits, as well as vessels, were positive for RB4CD12 (Figure 3B). Amyloid deposits and microvessels were also stained with RB4CD12 in AD hippocampus (Figure 3C). Interestingly, some pyramidal neurons in AD hippocampus showed intracellular granular staining (Figure 3D). These

intracellular staining signals were detected in a certain number of cells that were positive for hyperphosphorylated microtubule-associated protein tau as revealed by the AT180 monoclonal antibody (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

Expression of the RB4CD12 Epitope Borne in Molecules with 70–180 kDa Molecular Weights Is Upregulated in the Cortex of Tg2576 Mice

As an extension of the staining results in the mouse and human brain tissues, we wished to determine which proteins contain the RB4CD12 epitope and were differentially expressed in Tg2576 brains. We performed Western blotting for cortex samples, which were fractionated as TBS-insoluble/1% SDS-soluble. Four non-Tg and five Tg2576 mice (20 months old) were examined. Multiple bands were positive for RB4CD12 antibody in both non-Tg and Tg2576 (Figure 4A). We measured intensities of 460 kDa, 180 kDa, 120 kDa, and 100–70 kDa bands by densitometry. There was a 1.3-fold increase in the intensity of overall RB4CD12 recognition determinants in Tg2576 brain extracts compared with non-Tg controls (Figure 4B). Expression levels of RB4CD12 epitopes in bands of 180 kDa, 120 kDa, and 100–70 kDa were increased 1.1- to 1.5-fold in the cortex of Tg2576 mice (Figure 4B). There was no significant change in the intensity of bands of 460 kDa. Syndecan-3 and glypican-1 are HSPGs expressed in glial cells surrounding A β plaques of Tg2576 mice.²² To ascertain whether these proteins are HSPGs that contain the RB4CD12 epitope, we then analyzed their expression. Western blotting revealed the protein bands at 250 to 180 kDa for syndecan-3 and 60 kDa for glypican-1 in the cortex of Tg2576 mice (see Supplemental Figure S2A at <http://ajp.amjpathol.org>). Expression levels of these proteins in the Tg2576 cortex were comparable to those in non-Tg controls (see Supplemental Figure S2B at <http://ajp.amjpathol.org>).

Disaccharide Compositions of HS and Expression Profiles of HS Enzymes in the Cortex of Tg2576 and Postmortem AD

We performed structural analysis of HS chains extracted from mouse and human postmortem brains (Table 1). HS was isolated from the cortex of mice or postmortem human entorhinal cortex. HS was depolymerized into its constituent disaccharides by a mix of bacterial heparinases. The disaccharide compositions of the HS were determined by reversed-phase ion-pair chromatography. We found that the total HS contents and HS disaccharide compositions in vessel-enriched fractions ("vessel-enriched fr") and non-vessel associated fractions ("non-vasculature fr") were comparable between non-Tg and Tg2576 mice (Figure 4C). In human, the reduction in the proportion of non-sulfated disaccharides reached statistical significance. Total HS contents and percentages of other sulfated disaccharides were comparable between NDC and AD (Figure 4D). To understand possible mechanisms of upregulation of the RB4CD12 epitope in AD mouse brains, we measured mRNA levels of 10 HS modification enzymes by quantitative real time-PCR. HS enzymes include sulfotransferases and extracellular sulfatases. These enzymes are regarded as

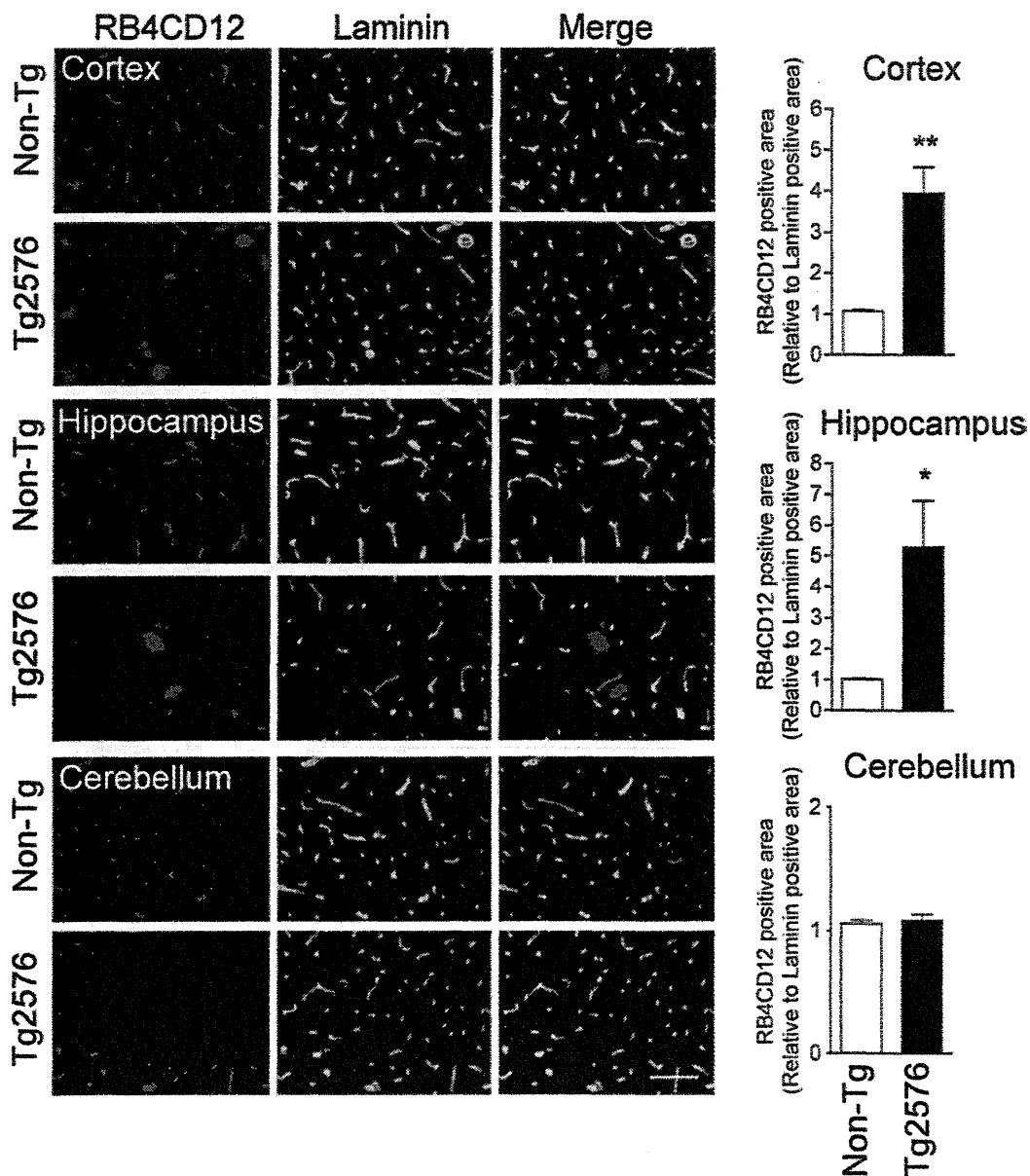


Figure 2. Quantification of the RB4CD12 epitope in nonvascular parenchyma in the brain of Tg2576 mice. Cryostat-cut brain sections of Tg2576 mice were stained with RB4CD12 (red) and anti-laminin (green) antibodies. Laminin is a marker for brain vessels. Nonvascular amyloid β plaques were stained with RB4CD12 antibody in the cortex and hippocampus. Graphs are of semiquantitative analysis of immunohistochemical pictures of RB4CD12 and laminin. RB4CD12-positive areas that were not colocalized with anti-laminin staining signals were calculated. * $P < 0.05$, ** $P < 0.01$.

key molecules in the regulation of sulfation of HS. The mRNA level of N-deacetylase/N-sulfotransferase 2 were significantly increased in Tg2576 (24%) (Figure 4E). The mRNA levels of Sulf-1 and Sulf-2 were comparable between non-Tg and Tg2576 mice (Figure 4E).

Sulf-1 and Sulf-2, Extracellular HS Sulfatases, Degrade the RB4CD12 Epitope Accumulated in Amyloid Plaques

Previously, we showed that the treatment of wild-type mouse brain sections with Sulf-1 or Sulf-2 greatly diminished the RB4CD12 epitope abundant in vasculature.⁸ To

determine whether the RB4CD12 epitope in amyloid plaques is susceptible to Sulf-1 and Sulf-2 and degraded by these enzymes, we treated cryo-cut brain sections of 18-month-old Tg2576 mice with recombinant Sulf-1, Sulf-2, or conditioned medium of MCF-7 breast cancer cells, which secrete native Sulf-2.²³ These treatments substantially reduced the RB4CD12 epitope in sections of Tg2576 brain *ex vivo* (Figure 5). A mixture of bacterial heparinases confirmed that the assay is suitable for *ex vivo* degradation of HS in brain sections and that the observed signals arose from HS (Figure 5). Anti- β staining signals that were colocalized with the RB4CD12 epitope were retained after Sulf treatment (Figure 5).

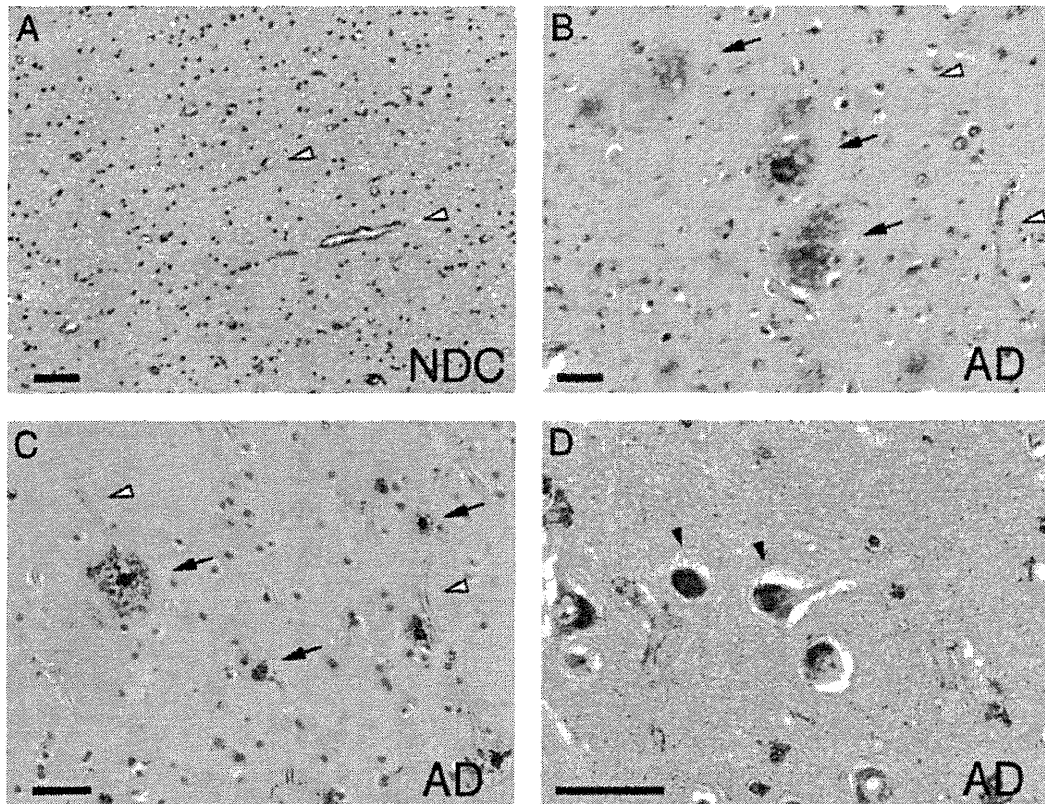


Figure 3. RB4CD12 epitope immunolocalizes in amyloid plaques in the brains of Alzheimer's disease patients. Immunoperoxidase staining for RB4CD12 (brown) in the entorhinal cortex (A, B) and hippocampus (C, D). **Open arrowheads** in A–C show vessel-staining signals in brains of non-demented control (NDC) and Alzheimer's disease (AD). In AD, amyloid deposits were also positive for RB4CD12 (**arrows** in B and C). Intracellular RB4CD12-staining signals are seen in some hippocampal neurons of AD (**arrowheads** in D). Scale bars: 50 μ m.

Discussion

In the present study, we showed that the RB4CD12 epitope is colocalized with amyloid plaques in brains of AD mouse models and patients with AD. Consistent with our previous report,⁹ the RB4CD12 epitope was also colocalized with laminin-positive vasculature in brains of mouse models of AD. Quantification analysis revealed that the non-vascular RB4CD12-positive area was increased in the cortex and hippocampus of Tg2576, J20, and T41 AD models. In the cerebellum, where no amyloid plaques were observed in these model mice, RB4CD12 staining was comparable to that in the non-Tg. Morphological alterations of the vasculature observed in the cortex and hippocampus of Tg2576 were consistent with the previous report that A β aggregates induce the structural and functional disruption of smooth muscle cells in the vasculature.²⁴ Results in aging brains of Tg2576 mice suggested that A β and the HS highly sulfated domains start accumulation at the same age. A β and other self-aggregating peptides share cationic motifs that may be involved in binding to the negative charges of sulfated glycosaminoglycan.^{25,26} HS and other glycosaminoglycan chains can stabilize mature fibrils against proteolytic degradation.²⁷ HS facilitates the formation of fibrils of amylin,¹⁷ apo-serum amyloid A,²⁸ α -synuclein,²⁹ prion protein,³⁰ muscle acylphosphatase,³¹ transthyretin,³² Tau,³³ and A β .^{34–36} *In vivo* fragmentation of heparan sul-

fate by heparanase overexpression could protect mice from amyloid protein A amyloidosis.³⁷ Importantly, the degree of sulfation is critical for enhancement of fibrillogenesis of A β .³⁵ Pathological effects of heparin in A β aggregation assays are dependent on sulfate moieties at N- and O-positions.³⁸ Our findings of selective accumulation of the RB4CD12 epitope in amyloid plaques suggest that highly sulfated domains of HS could play an important role in the progression of A β deposition. HSPG facilitates cerebral amyloid deposition, which can be induced exogenously in a rat model.¹⁵ Highly sulfated HS chains could be one candidate for heat-resistant materials present in the brain extract that are essential for exogenous induction of cerebral β -amyloidogenesis in mouse models.³⁹ Recently, Timmer et al⁴⁰ demonstrated that only a minimal number of A β plaques (~30%) were co-stained with the epitope of JM403, an anti-HS antibody, in aging brains of APPswe/PS1dE9 model mice. JM403 detects HS subdomains containing the positively charged disaccharide [β -glucuronic/iduronic acid-N-unsubstituted glucosamine].⁴¹ Future studies may reveal differential contribution of HS subdomains composed of specific disaccharide structures to AD pathogenesis. Possible involvement of the RB4CD12 epitope existing in laminin-positive vasculature in AD pathogenesis should also be clarified in the future. Interestingly, intraneuronal RB4CD12 staining was observed in the

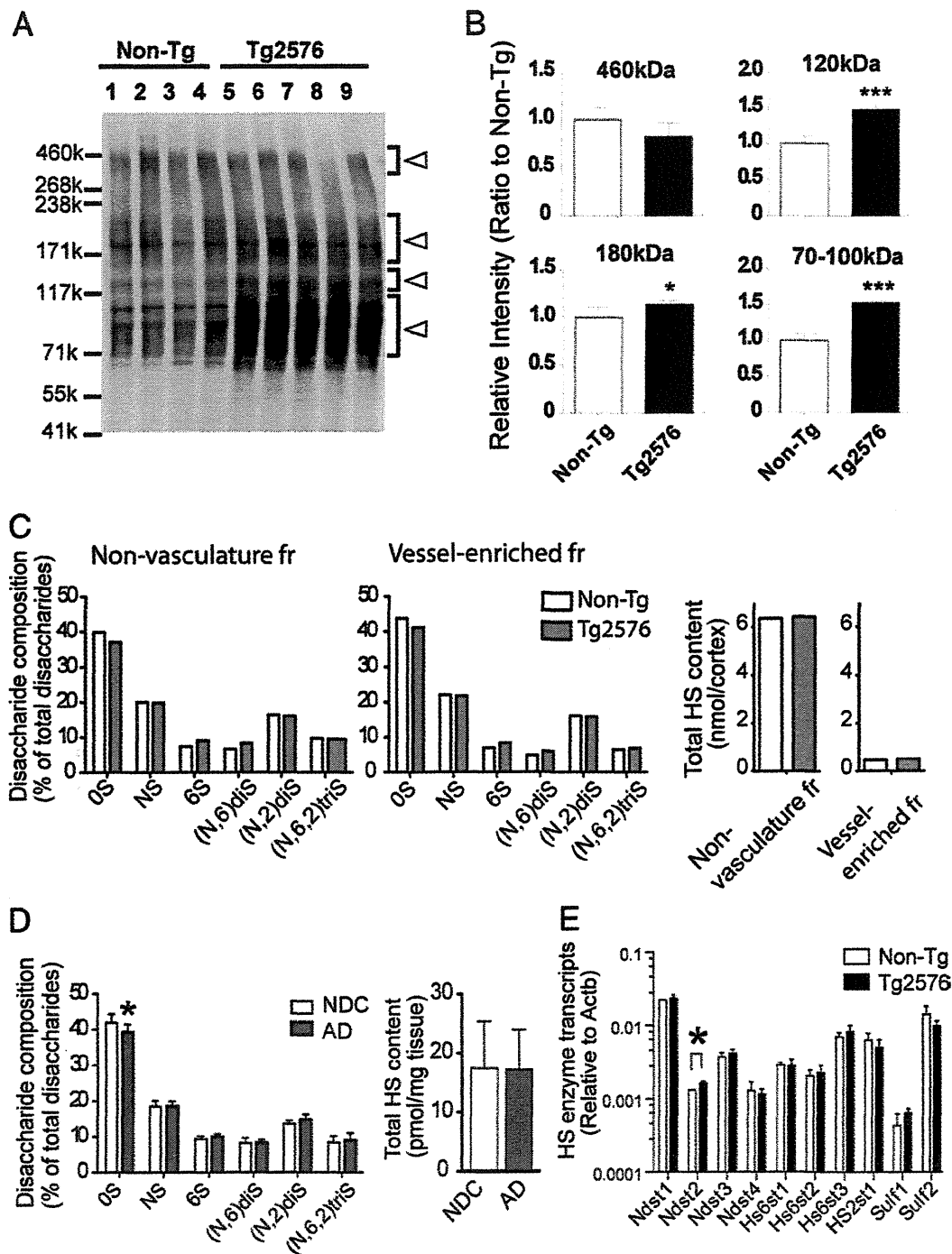


Figure 4. Immunoblotting analysis of the RB4CD12 epitope, disaccharide composition analysis of Heparan sulfate (HS) and quantitative real-time PCR analysis of HS enzymes in the brain of Tg2576 mice. **A:** Tris-buffered saline (TBS)-insoluble/1% SDS-soluble fractions were prepared from tissue homogenates of four 20-month-old Tg2576 (lanes 1–4) and five 20-month-old non-Tg (lanes 5–9) cortices. Immunoblot with RB4CD12 was performed as described in *Materials and Methods*. **B:** Relative intensities of bands with molecular weights of 460 kDa, 180 kDa, 120 kDa, and 70 to 100 kDa indicated by open arrowheads in (A) were measured. **C:** High performance liquid chromatography analysis determined non-sulfated (OS), monosulfated (NS, 6S), disulfated (N,6)diS and trisulfated (N,6,2)triS disaccharide compositions in non-vasculature fractions and vessel-enriched fractions of 18-month-old non-Tg and Tg2576 cortices. The level of total HS was determined by summing amounts of all disaccharides detected in each fraction. The values are representative of two independent experiments. **D:** HS disaccharide compositions and the level of total HS in the entorhinal cortex of non-demented control (NDC) ($n = 8$) and Alzheimer's disease (AD) ($n = 8$) postmortem brains were determined. **E:** Total-RNA from the cerebral cortices of 18-month-old Non-Tg ($n = 3$) and Tg2576 mice ($n = 3$) were prepared and tested. mRNA levels of 10 HS modification enzymes were determined by quantitative real-time PCR. * $P < 0.05$, *** $P < 0.001$.

hippocampus of AD patients. Microtubule-associated protein Tau is the major protein subunit of intraneuronal neurofibrillary tangles, another neuropathological hallmark of AD.⁴² It has been shown that Tau and HS

coexist in nerve cells with overt neurofibrillary lesion.³³ The filamentous structures induced by heparin are structurally similar to those found in Alzheimer's disease.^{43–45} We have shown that some of RB4CD12

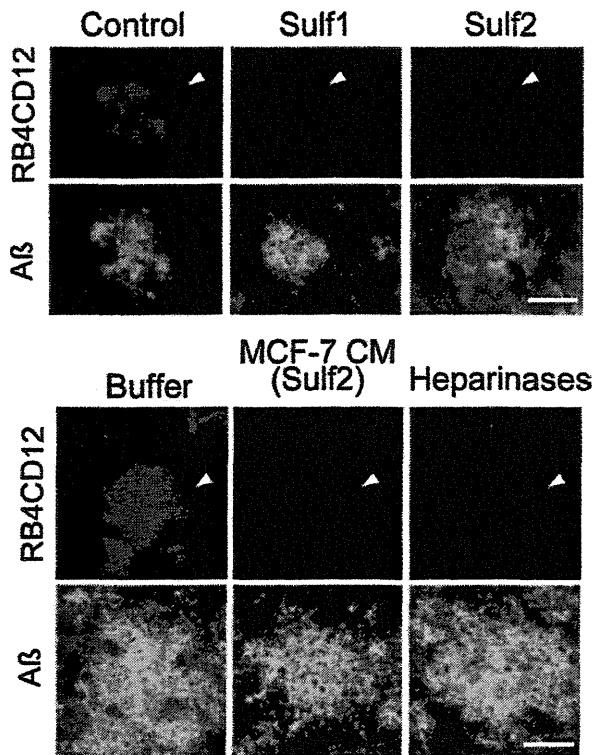


Figure 5. The RB4CD12 epitope in amyloid plaques of Tg2576 mouse brains is degraded *ex vivo* by Sulf-1, Sulf-2 and conditioned medium of Sulf-2-expressing cells. Cryostat-cut consecutive sections of 18-month-old Tg2576 mouse brains were incubated overnight with recombinant human Sulf-1 and Sulf-2 prepared from CM of transfected HEK293 cells (Sulf1, Sulf2), buffer only (Buffer), or CM of MCF-7 human breast cancer cells (MCF-7 CM).^{8,23} The Ni-NTA resin-bound materials that were prepared from HEK293 cells transfected with the empty vector were eluted and used (control). A mix of bacterial heparinases (heparinases) served as a positive control. RB4CD12 binding was visualized using a Cy3-conjugated anti-VSV tag antibody (red). Treated sections were co-stained with 82E1 anti-A β antibody (green). The data are representative of two independent experiments. **Arrowheads** indicate amyloid plaques. Scale bars: 20 μ m.

staining signals were found in cells that were stained with AT180, an antibody against hyperphosphorylated tau. Our results suggest that highly sulfated domains of HS might play a role in the formation of neurofibrillary tangles.

In immunoblots of brain lysates with the RB4CD12 antibody, we detected several RB4CD12-positive bands in non-Tg and Tg2576 mouse brains and found that 180 kDa, 120 kDa, and 100–70 kDa bands were upregulated in the cortex of Tg2576 mice. There were no significant changes in the intensities of 460 kDa bands. Our previous results showed that the RB4CD12 epitope is abundant in the basement membrane of the brain vessels and that the RB4CD12-positive bands were predominantly 460 kDa bands in brain vessel fractions.⁹ In our immunohistochemical studies, non-vascular RB4CD12 staining was increased in Tg2576 mice. These results suggest that upregulation of 180 kDa, 120 kDa, and 100–70 kDa bands could contribute to the RB4CD12 staining colocalized with amyloid plaques in Tg2576 mouse brain. Several HSPGs are known to be localized in amyloid plaques.^{14,46–49} Because of the high molecular weight (>210 kDa) of agrin and perlecan, it is conceivable that the observed signals in immunoblots might have arisen from other molecules. Syndecan-3 and glypican-1 in glial

cells were identified as molecules associated with A β deposits.²² Our Western blotting results suggested that syndecan-3 with the molecular weights of 180 to 250 kDa could be an HSPG that possesses the RB4CD12 epitope. However, we cannot rule out the possibility that degradation products of agrin or perlecan could harbor the RB4CD12 epitope observed in amyloid plaques. We should also pay attention to possible accumulation of HS degradation products catalyzed by nitric oxide.⁵⁰

Unexpectedly, the trisulfated disaccharide composition was not increased in either Tg2576 or human post-mortem AD brains. The mechanisms underlying the accumulation of the RB4CD12 highly sulfated domains within HS polysaccharides in non-vasculature amyloid plaques are not clear. There are two possibilities to explain the mechanisms. First, the N-sulfation of glucosamine residues is the initial HS sulfation and the N-sulfated domains are primary sites for further modification.⁵¹ Consecutive occurrence of N-sulfation could be attributable to the formation of trisulfated disaccharide clusters, namely, highly sulfated domains, within HS chains in non-vasculature spaces. Second, translocation of HS that contains the RB4CD12 highly sulfated domains between brain vasculature and non-vasculature could be an explanation for the accumulation of the RB4CD12 epitope in Tg2576 brain parenchyma. Our findings that comparable levels of disaccharide compositions and HS contents in vessel-enriched fractions and non-vasculature fractions in the cortex of Tg2576 were shown and that the mRNA level of N-deacetylase/N-sulfotransferase 2 was increased in the cortex of Tg2576 mouse could support the former possibility. A previous study by Lindahl et al⁵² showed altered distribution of N-sulfated glucosamine residues within HS extracted from postmortem AD brain. Highly N-sulfated HS may be involved in the initiation of the aggregation process of A β in AD brains.⁵³ These studies also support the former possibility as an explanation of the mechanisms of accumulation of RB4CD12-positive highly sulfated domains in A β plaques. We cannot rule out the possibility that the RB4CD12 epitope is a minor component and that the structural analysis we have performed might not fully detect the minor change. Quantitative analysis for the RB4CD12-positive HS in the cortex would make advances in the study of the mechanisms.

Herein, we found that the RB4CD12 epitope accumulated in amyloid plaques can be degraded by Sulf-1 and Sulf-2 *ex vivo*. It was suggested that the RB4CD12 highly sulfated domains are localized at the surface of amyloid plaques, as these HS degrading enzymes could access and efficiently degrade the epitope. Although the RB4CD12 epitope in amyloid plaques was degraded by the Sulfs, substantial amounts of A β were retained in these plaques. This result suggests that the highly sulfated domains of HS universally associated with amyloid deposits in the brain. Accumulation of the RB4CD12 epitope in amyloid plaques could induce excessive entrapment of growth factors at amyloid plaques, which might lead to an imbalance in homeostasis of the brain microenvironment. Increasing evidence points to vascular damage as an early contributor in Alzheimer pathology.^{54,55} A recent study suggested that angiogenesis

might be impaired in AD model mice,⁵⁶ despite the fact that the levels of pro-angiogenic growth factors (eg, vascular endothelial growth factor [VEGF]) are elevated in AD brains.^{57–59} VEGF binds to immobilized heparin and can be stored in the extracellular space by binding to HS and HSPG.^{23,60} Heparin-bound VEGF is mobilized by the action of Sulf-2, which exerts pro-angiogenic activity.^{23,61} VEGF is found to be associated with amyloid plaques in AD, but not non-AD brain.⁶² Our results also suggested that the highly sulfated domains could be involved in sequestration of VEGF within amyloid plaques and vascular damage in AD through perturbation in the supply of pro-angiogenic growth factors. Aberrant angiogenesis could induce neurovascular uncoupling, which ultimately leads to synaptic dysfunction.⁶³ In summary, we provide evidence that highly sulfated domains recognized by RB4CD12 accumulated in amyloid plaques of brains of AD model mice and patients with AD. Further studies to investigate the roles of the highly sulfated HS domains with special regard to angiogenesis in AD pathology will be needed.

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References

1. Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M: Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 1999, 68:729–777
2. Esko JD, Lindahl U: Molecular diversity of heparan sulfate. *J Clin Invest* 2001, 108:169–173
3. Gallagher JT: Heparan sulfate: growth control with a restricted sequence menu. *J Clin Invest* 2001, 108:357–361
4. Nakato H, Kimata K: Heparan sulfate fine structure and specificity of proteoglycan functions. *Biochim Biophys Acta* 2002, 1573:312–318
5. Parish CR: The role of heparan sulphate in inflammation. *Nat Rev Immunol* 2006, 6:633–643
6. Bishop JR, Schuksz M, Esko JD: Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature* 2007, 446:1030–1037
7. Dennissen MA, Jenniskens GJ, Pieffers M, Versteeg EM, Petitou M, Veerkamp JH, van Kuppevelt TH: Large, tissue-regulated domain diversity of heparan sulfates demonstrated by phage display antibodies. *J Biol Chem* 2002, 277:10982–10986
8. Hossain MM, Hosono-Fukao T, Tang R, Sugaya N, van Kuppevelt TH, Jenniskens GJ, Kimata K, Rosen SD, Uchimura K: Direct detection of HSulf-1 and HSulf-2 activities on extracellular heparan sulfate and their inhibition by PI-88. *Glycobiology* 2010, 20:175–186
9. Hosono-Fukao T, Ohtake-Niimi S, Nishitsuji K, Hossain MM, van Kuppevelt TH, Michikawa M, Uchimura K: RB4CD12 epitope expression and heparan sulfate disaccharide composition in brain vasculature. *J Neurosci Res* 2011, 89:1840–1848
10. Esko JD, Selleck SB: Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu Rev Biochem* 2002, 71:435–471
11. Saad OM, Ebel H, Uchimura K, Rosen SD, Bertozzi CR, Leary JA: Compositional profiling of heparin/heparan sulfate using mass spectrometry: assay for specificity of a novel extracellular human endosulfatase. *Glycobiology* 2005, 15:818–826
12. Morimoto-Tomita M, Uchimura K, Werb Z, Hemmerich S, Rosen SD: Cloning and characterization of two extracellular heparin-degrading

- endosulfatases in mice and humans. *J Biol Chem* 2002, 277:49175–49185
13. Snow AD, Mar H, Nochlin D, Kimata K, Kato M, Suzuki S, Hassell J, Wight TN: The presence of heparan sulfate proteoglycans in the neuritic plaques and congophilic angiopathy in Alzheimer's disease. *Am J Pathol* 1988, 133:456–463
14. Snow AD, Sekiguchi RT, Nochlin D, Kalaria RN, Kimata K: Heparan sulfate proteoglycan in diffuse plaques of hippocampus but not of cerebellum in Alzheimer's disease brain. *Am J Pathol* 1994, 144:337–347
15. Snow AD, Sekiguchi R, Nochlin D, Fraser P, Kimata K, Mizutani A, Arai M, Schreier WA, Morgan DG: An important role of heparan sulfate proteoglycan (Perlecan) in a model system for the deposition and persistence of fibrillar A beta-amyloid in rat brain. *Neuron* 1994, 12:219–234
16. van Horsen J, Wesseling P, van den Heuvel LP, de Waal RM, Verbeek MM: Heparan sulphate proteoglycans in Alzheimer's disease and amyloid-related disorders. *Lancet Neurol* 2003, 2:482–492
17. Watson DJ, Lander AD, Selkoe DJ: Heparin-binding properties of the amyloidogenic peptides Abeta and amylin. Dependence on aggregation state and inhibition by Congo red. *J Biol Chem* 1997, 272:31617–31624
18. Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G: Correlative memory deficits. A beta elevation, and amyloid plaques in transgenic mice. *Science* 1996, 274:99–102
19. Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L: High-level neuronal expression of abeta 1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J Neurosci* 2000, 20:4050–4058
20. Rockenstein E, Mallory M, Mante M, Sisk A, Masliah E: Early formation of mature amyloid-beta protein deposits in a mutant APP transgenic model depends on levels of A beta(1–42). *J Neurosci Res* 2001, 66:573–582
21. Jenniskens GJ, Oosterhof A, Brandwijk R, Veerkamp JH, van Kuppevelt TH: Heparan sulfate heterogeneity in skeletal muscle basal lamina: demonstration by phage display-derived antibodies. *J Neurosci* 2000, 20:4099–4111
22. O'Callaghan P, Sandwall E, Li JP, Yu H, Ravid R, Guan ZZ, van Kuppevelt TH, Nilsson LN, Ingelsson M, Hyman BT, Kalimo H, Lindahl U, Lannfelt L, Zhang X: Heparan sulfate accumulation with Abeta deposits in Alzheimer's disease and Tg2576 mice is contributed by glial cells. *Brain Pathol* 2008, 18:548–561
23. Uchimura K, Morimoto-Tomita M, Bistrup A, Li J, Lyon M, Gallagher J, Werb Z, Rosen SD: HSulf-2, an extracellular endoglucosaminase-6-sulfatase, selectively mobilizes heparin-bound growth factors and chemokines: effects on VEGF, FGF-1, and SDF-1. *BMC Biochem* 2006, 7:2
24. Christie R, Yamada M, Moskowitz M, Hyman B: Structural and functional disruption of vascular smooth muscle cells in a transgenic mouse model of amyloid angiopathy. *Am J Pathol* 2001, 158:1065–1071
25. Diaz-Nido J, Wandosell F, Avila J: Glycosaminoglycans and beta-amyloid, prion and tau peptides in neurodegenerative diseases. *Peptides* 2002, 23:1323–1332
26. McLaurin J, Fraser PE: Effect of amino-acid substitutions on Alzheimer's amyloid-beta peptide-glycosaminoglycan interactions. *Eur J Biochem* 2000, 267:6353–6361
27. Gupta-Bansal R, Frederickson RC, Brunden KR: Proteoglycan-mediated inhibition of A beta proteolysis. A potential cause of senile plaque accumulation. *J Biol Chem* 1995, 270:18666–18671
28. Ancsin JB, Kisilevsky R: The heparin/heparan sulfate-binding site on apo-serum amyloid A. Implications for the therapeutic intervention of amyloidosis. *J Biol Chem* 1999, 274:7172–7181
29. Cohlberg JA, Li J, Uversky VN, Fink AL: Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from alpha-synuclein in vitro. *Biochemistry* 2002, 41:1502–1511
30. Supattapone S: Prion protein conversion in vitro. *J Mol Med* 2004, 82:348–356
31. Motamedi-Shad N, Monsellier E, Torrasa S, Relini A, Chiti F: Kinetic analysis of amyloid formation in the presence of heparan sulfate: faster unfolding and change of pathway. *J Biol Chem* 2009, 284:29921–29934

32. Noborn F, O'Callaghan P, Hermansson E, Zhang X, Ancsin JB, Damas AM, Dacklin I, Presto J, Johansson J, Saraiva MJ, Lundgren E, Kisilevsky R, Westermark P, Li JP: Heparan sulfate/heparin promotes transthyretin fibrillization through selective binding to a basic motif in the protein. *Proc Natl Acad Sci USA* 2011, 108:5584–5589
33. Goedert M, Jakes R, Spillantini MG, Hasegawa M, Smith MJ, Crowther RA: Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. *Nature* 1996, 383:550–553
34. McLaurin J, Franklin T, Zhang X, Deng J, Fraser PE: Interactions of Alzheimer amyloid-beta peptides with glycosaminoglycans effects on fibril nucleation and growth. *Eur J Biochem* 1999, 266:1101–1110
35. Castillo GM, Lukito W, Wight TN, Snow AD: The sulfate moieties of glycosaminoglycans are critical for the enhancement of beta-amyloid protein fibril formation. *J Neurochem* 1999, 72:1681–1687
36. Valle-Delgado JJ, Alfonso-Prieto M, de Groot NS, Ventura S, Samitier J, Rovira C, Fernandez-Busquets X: Modulation of Abeta42 fibrillogenesis by glycosaminoglycan structure. *FASEB J* 2010, 24:4250–4261
37. Li JP, Galvis ML, Gong F, Zhang X, Zcharia E, Metzger S, Vlodavsky I, Kisilevsky R, Lindahl U: In vivo fragmentation of heparan sulfate by heparanase overexpression renders mice resistant to amyloid protein A amyloidosis. *Proc Natl Acad Sci USA* 2005, 102:6473–6477
38. Timmer NM, Schirris TJ, Bruinsma IB, Otte-Holler I, van Kuppevelt TH, de Waal RM, Verbeek MM: Aggregation and cytotoxic properties towards cultured cerebrovascular cells of Dutch-mutated Abeta40 (DAbeta(1–40)) are modulated by sulfate moieties of heparin. *Neurosci Res* 2010, 66:380–389
39. Meyer-Luehmann M, Coomaraswamy J, Bolmont T, Kaeser S, Schaefer C, Kilger E, Neuenschwander A, Abramowski D, Frey P, Jaton AL, Vigouret JM, Paganetti P, Walsh DM, Mathews PM, Ghiso J, Staufenbiel M, Walker LC, Jucker M: Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host. *Science* 2006, 313:1781–1784
40. Timmer NM, Herbert MK, Kleinovink JW, Kiliaan AJ, De Waal RM, Verbeek MM: Limited expression of heparan sulphate proteoglycans associated with Abeta deposits in the APP^{swe}/PS1^{dE9} mouse model for Alzheimer's disease. *Neuropathol Appl Neurobiol* 2010, 36:478–486
41. van den Born J, Salmivirta K, Henttinen T, Ostman N, Ishimaru T, Miyaura S, Yoshida K, Salmivirta M: Novel heparan sulfate structures revealed by monoclonal antibodies. *J Biol Chem* 2005, 280:20516–20523
42. Skovronsky DM, Lee VM, Trojanowski JQ: Neurodegenerative diseases: new concepts of pathogenesis and their therapeutic implications. *Annu Rev Pathol* 2006, 1:151–170
43. Kuret J, Chirita CN, Congdon EE, Kannanayakal T, Li G, Necula M, Yin H, Zhong Q: Pathways of tau fibrillization. *Biochim Biophys Acta* 2005, 1739:167–178
44. Perez M, Valpuesta JM, Medina M, Montejo de Garcini E, Avila J: Polymerization of tau into filaments in the presence of heparin: the minimal sequence required for tau-tau interaction. *J Neurochem* 1996, 67:1183–1190
45. Zhu HL, Fernandez C, Fan JB, Shewmaker F, Chen J, Minton AP, Liang Y: Quantitative characterization of heparin binding to Tau protein: implication for inducer-mediated Tau filament formation. *J Biol Chem* 2010, 285:3592–3599
46. Van Gool D, David G, Lammens M, Baro F, Dom R: Heparan sulfate expression patterns in the amyloid deposits of patients with Alzheimer's and Lewy body type dementia. *Dementia* 1993, 4:308–314
47. Donahue JE, Berzin TM, Rafii MS, Glass DJ, Yancopoulos GD, Fallon JR, Stopa EG: Agrin in Alzheimer's disease: altered solubility and abnormal distribution within microvasculature and brain parenchyma. *Proc Natl Acad Sci USA* 1999, 96:6468–6472
48. Verbeek MM, Otte-Holler I, van den Born J, van den Heuvel LP, David G, Wesseling P, de Waal RM: Agrin is a major heparan sulfate proteoglycan accumulating in Alzheimer's disease brain. *Am J Pathol* 1999, 155:2115–2125
49. van Horssen J, Otte-Holler I, David G, Maat-Schieman ML, van den Heuvel LP, Wesseling P, de Waal RM, Verbeek MM: Heparan sulfate proteoglycan expression in cerebrovascular amyloid beta deposits in Alzheimer's disease and hereditary cerebral hemorrhage with amyloidosis (Dutch) brains. *Acta Neuropathol* 2001, 102:604–614
50. Cappai R, Cheng F, Ciccotosto GD, Needham BE, Masters CL, Multhaup G, Fransson LA, Mani K: The amyloid precursor protein (APP) of Alzheimer disease and its paralog, APLP2, modulate the Cu/Zn-Nitric Oxide-catalyzed degradation of glypican-1 heparan sulfate in vivo. *J Biol Chem* 2005, 280:13913–13920
51. Carlsson P, Presto J, Spillmann D, Lindahl U, Kjellen L: Heparin/heparan sulfate biosynthesis: processive formation of N-sulfated domains. *J Biol Chem* 2008, 283:20008–20014
52. Lindahl B, Eriksson L, Lindahl U: Structure of heparan sulphate from human brain, with special regard to Alzheimer's disease. *Biochem J* 1995, 306 (Pt 1):177–184
53. Bruinsma IB, te Riet L, Gevers T, ten Dam GB, van Kuppevelt TH, David G, Kusters B, de Waal RM, Verbeek MM: Sulfation of heparan sulfate associated with amyloid-beta plaques in patients with Alzheimer's disease. *Acta Neuropathol* 2010, 119:211–220
54. Bailey TL, Rivara CB, Rocher AB, Hof PR: The nature and effects of cortical microvascular pathology in aging and Alzheimer's disease. *Neurosci Res* 2004, 26:573–578
55. Meyer EP, Ulmann-Schuler A, Staufenbiel M, Krucker T: Altered morphology and 3D architecture of brain vasculature in a mouse model for Alzheimer's disease. *Proc Natl Acad Sci USA* 2008, 105:3587–3592
56. Paris D, Ganey N, Banasiak M, Laporte V, Patel N, Mullan M, Murphy SF, Yee GT, Bachmeier C, Ganey C, Beaulieu-Abdelahad D, Mathura VS, Brem S: Impaired orthotopic glioma growth and vascularization in transgenic mouse models of Alzheimer's disease. *J Neurosci* 2010, 30:11251–11258
57. Kalaria RN, Cohen DL, Premkumar DR, Nag S, LaManna JC, Lust WD: Vascular endothelial growth factor in Alzheimer's disease and experimental cerebral ischemia. *Brain Res Mol Brain Res* 1998, 62:101–105
58. Tarkowski E, Issa R, Sjogren M, Wallin A, Blennow K, Tarkowski A, Kumar P: Increased intrathecal levels of the angiogenic factors VEGF and TGF-beta in Alzheimer's disease and vascular dementia. *Neurobiol Aging* 2002, 23:237–243
59. Siedlak SL, Cras P, Kawai M, Richey P, Perry G: Basic fibroblast growth factor binding is a marker for extracellular neurofibrillary tangles in Alzheimer disease. *J Histochem Cytochem* 1991, 39:899–904
60. Iozzo RV: Matrix proteoglycans: from molecular design to cellular function. *Annu Rev Biochem* 1998, 67:609–652
61. Morimoto-Tomita M, Uchimura K, Bistrup A, Lum DH, Egeblad M, Boudreau N, Werb Z, Rosen SD: Sulf-2, a proangiogenic heparan sulfate endosulfatase, is upregulated in breast cancer. *Neoplasia* 2005, 7:1001–1010
62. Ryu JK, Cho T, Choi HB, Wang YT, McLarnon JG: Microglial VEGF receptor response is an integral chemotactic component in Alzheimer's disease pathology. *J Neurosci* 2009, 29:3–13
63. Zlokovic BV: Neurovascular mechanisms of Alzheimer's neurodegeneration. *Trends Neurosci* 2005, 28:202–208

Chapter VII

Regulation by FGF-1 of apoE/HDL Generation in Astrocytes

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Abstract

The neural cells in the central nervous system (CNS) are segregated by the blood-brain barrier (BBB) from cholesterol transporters such as low density lipoproteins (LDL) and very low density lipoproteins (VLDL) in human blood plasma, although the CNS is an organ to require cholesterol at very high level. Therefore, cholesterol homeostasis in the CNS is surely controlled by the brain-specific cholesterol transport system. Apolipoprotein E (apoE) produced by astrocytes is a most abundant apolipoprotein in the cerebrospinal fluid (CSF). Astrocytes generate cholesterol-rich high density lipoprotein (apoE/HDL) by using the endogenous apoE and phospholipid-rich and cholesterol-poor HDL (apoA-I/HDL) through the interaction with exogenous apoA-I which is probably produced by brain endothelial cells.

The secretion of endogenous apoE from rat astrocytes is started within 30 min after the initiation of biosynthesis. The apoE secreted from rat astrocytes uses pre-synthesized cholesterol rather than newly-synthesized cholesterol for biogenesis of HDL, although exogenous human apoE mediates effluxes of both pre-synthesized and newly synthesized cholesterols as well as exogenous cyclodextrin. This suggests that astrocytes secrete hardly endogenous apoE as a lipid-free apoE.

Rat astrocytes cultured for one month (long-term cultured astrocytes) enhance significantly generation of apoE/HDL dependently on fibroblast growth factor 1 (FGF-1) produced by astrocytes in the manner of apparent autocrine action. The release of FGF-1 is likely provoked by oxidative stress involved in long-term cultured stress. Purified FGF-1 enhances the apoE/HDL generation of astrocytes accompanied with enhancing syntheses of both apoE and cholesterol. FGF-1 increases cholesterol synthesis through the activation of MAP kinase cascade in astrocytes, stimulates intracellular apoE

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transport through PI3-kinase/Akt pathway and enhances apoE mRNA expression through increasing mRNA expression of LXR α .

The mechanism underlying FGF-1 release from astrocytes and subsequent generation of apoE/HDL are important issues to be addressed to understand the role of FGF-1 in damaged brains, including brain injury, brain inflammation, or neurodegenerative diseases. The regulation by FGF-1 of apoE/HDL generation under the stress-conditions is mainly argued on the base of our experimental results including recently obtained and unpublished several data in this chapter.

Introduction

Cholesterol is an important component lipid of cell membrane. The hydroxyl group of cholesterol molecule is located near the hydrophilic head of phospholipid molecule and the flat steroid structure is filled up among hydrocarbon chains of phospholipids molecules. Such a feature of chemical structure in cholesterol molecule is useful for regulation of fluidity and viscosity of cell membrane. Cholesterol, furthermore, has a high affinity for sphingomyelin and constructs a membrane lipid raft, a cholesterol- and sphingomyelin-rich domain, to offer a platform for molecules participating in signal transductions in the plasma membrane.

The CNS requires highly cholesterol, because neural cells such as neurons, astrocytes, oligodendroglia and microglia own complicated morphology with huge amount of plasma membrane and oligodendroglia organize myelin structure. Indeed, the CNS is a cholesterol-rich organ, in which approximately 20 - 25 % of total body cholesterol is localized to maintain functions and viability of neural cells [1]. The BBB, however, segregates neurons and glia in the brain from the systemic circulation and a major lipid transporter in human blood plasma, such as LDL. Therefore there is a unique and specific system for cholesterol delivery to neural cells to maintain cholesterol homeostasis in the brain. Lipoproteins found in the CSF are all HDL but not LDL or VLDL. Apolipoproteins identified in HDL of the CSF are mainly apoE and apoA-I in addition to minor apolipoproteins such as apolipoproteins A-IV, D and J [4,30]. The apoE produced and secreted by astrocytes functions dominantly in intercellular cholesterol transport between neural cells in the brain. The apoE appears to be un-exchangeable with the plasma apoE, because the phenotype of apoE in the CNS of recipient remains unchanged even after liver transplantation [36]. Therefore, the intercellular cholesterol transport in CNS is thought to be mediated by the HDL generated within the brain.

FGF-1 (acidic FGF, heparin-binding growth factor 1, HBGF-1) is one of members of FGF family and stimulates proliferation and differentiation of mesodermal and neuroectodermal cells as well as FGF-2 (basic FGF) [8,33]. FGF-1 isolated from neural tissue as a Mr 16,000 – 18,000 polypeptide is 55% identical in amino acid sequence with FGF-2. Many kinds of cell species produce and release FGF-1 in addition to FGF-2 at low level. In the brain, both FGF-1 and FGF-2 are distributed exhibiting the different localization. FGF-1 is known to be localized in all motor neurons and primary sensory neurons in the mesencephalon and much less in a small number of glial cells of white matter [9,10]. FGF-2 is mainly expressed in astrocytes and pyramidal neurons of the hippocampus [9]. FGF-1 appears to contribute to neuron survival and neurite outgrowth in vitro and in vivo [18,49]. In