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認知症対策総合研究事業

アルツハイマー病の新規細胞医薬開発に関する臨床応用研究

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研究代表者　内村　健治

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厚生労働科学研究費補助金（認知症対策総合研究事業）
総括研究報告書

アルツハイマー病の新規細胞医薬開発に関する臨床応用研究

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研究要旨：　骨髄由来ミクログリア細胞がアルツハイマー病（AD）病態に伴って脳内へ移行し、神経毒性アミロイドβタンパク（Aβ）を積極的に除去していることがADモデルマウスを用いて国内外で明らかにされている。しかしながらその脳内移行のメカニズムは不明である。申請者は以前、当該脳移行性細胞の脳内浸潤におけるセレクチン-糖鎖の分子メカニズムの重要性をADモデルマウスにより明らかにした。本申請研究はADモデルマウスで得られた知見および結果をヒト臨床サンプルの使用による臨床研究へ応用するために立案された。AD 治療法開発における問題の一つは遺伝子等の効率的な脳内動員法が確立していないことである。本申請研究は脳移行性細胞を利用した細胞医薬によりこの問題を解決し、AD 新規治療法開発の技術基盤の提供を目的とする。本研究者らはヒト骨髄由来細胞株のAD病態脳における動態を生体内ビデオ蛍光顕微鏡技術で解析した。ヒト骨髄由来細胞株がモデルマウス脳血管内でローリングおよび接着を示す結果を得た。また、当該細胞でセレクチンリガンド糖鎖の発現が観察された。一方、医療法人さわらび会福祉村病院 赤津裕康研究協力者より提供された非認知症８例、AD８例のヒト剖検脳サンプルにおけるセレクチンおよびリガンド糖鎖の発現変動を解析した。認知機能の中枢である海馬と共に提供を受けた嗅内皮質に関して発現解析を行った（計３２採取サンプル）。その結果、非認知症対照群に比べAD脳嗅内皮質でセレクチン分子の発現上昇が観察された。神経毒性重合Aβの除去を亢進させることが期待される細胞外スルファターゼ遺伝子をヒト細胞で発現し得るレンチウィルスベクターに組み込み、ウィルスを使用した遺伝子発現システムの開発に成功した。当該遺伝子を脳移行性細胞に導入しAD病態個体に投与後、脳病理変化を観察する研究への応用が期待された。ヒト臨床サンプルを用いた本申請研究は人権保護および個人情報保護に最大限の注意を払い、当該機関の倫理委員会による厳正中立な審査・承認を受けた。本研究の技術基盤により認知症を最小限に抑える研究成果が予想されその波及効果として、付随する介護負担の軽減が社会的に期待された。

Ａ．研究目的

超高齢化社会を迎えた我が国においてアルツハイマー病（AD）は増加の一途をたどっており、その治療法の確立は国民が強く求めるものとなっている。ADは神経毒性を引き起こす重合アミロイドβタンパク（Aβ）の沈着が脳内で生成され、神経細胞死やシナプスの機能障害・脱落が生じることにより認知症が発症すると主に考えられている。本研究は脳移行性細胞を重合 Aβ分解／阻止分子の脳内搬送体として利用し、AD 治療における細胞医薬技術基盤の確立を目指す。骨髄単球由来ミクログリアがAD病態に伴い脳内に浸潤することがモデルマウスを用いた解析により報告されている（Simard et al., Neuron, 2006; El Khoury et al., Nat Med 2007 など）。脳内へ移行した骨髄由来ミクログリア細胞は神経毒性 Aβを積極的に除去していることが国内外で明らかになってきた。しかしながらその脳内移行のメカニズムは不明である。申請者は以前、

末梢投与マウスミクログリア細胞の脳内動態を生体内ビデオ蛍光顕微鏡によりイメージング解析することに成功した。すなわち、当該脳移行性細胞の脳内浸潤におけるセレクチン-糖鎖の分子メカニズムの重要性をADモデルマウスにより明らかにした。本申請研究はADモデルマウスで得られた知見および結果をヒト臨床サンプルの使用による臨床研究へ応用するために立案された。AD治療法開発における問題の一つは遺伝子等の効率的な脳内動員法が確立していないことである。本申請研究は脳移行性細胞を利用した細胞医薬によりこの問題を解決し、AD新規治療法開発の技術基盤を提供することを目的とする。

B．研究方法

本研究は研究代表者である申請者が研究総括、実験計画の立案および遂行を行った。リサーチレジデント１名は生体内ビデオ蛍光顕微鏡を用いた脳内細胞イメージング解析および組織染色を研究協力した。医療法人さわらび会福祉村病院 長寿医学研究所 赤津裕康副所長よりヒト剖検脳の試料提供と研究協力を受けた。申請者は生体内ビデオ蛍光顕微鏡を本研究室に設置し末梢投与マウス骨髄由来ミクログリア細胞のADモデルマウス脳内におけるローリングおよび接着のイメージング解析に成功した。この成果をヒト骨髄由来細胞へ応用した。ヒト骨髄由来細胞は市販のヒト細胞株U937を用いた。ADモデルマウスは加齢育成したTg2576またはJ20マウスを使用した。アルツハイマー病および非認知症対照群でそれぞれ８例の剖検脳凍結サンプル（海馬および嗅内皮質）の提供を受けた。セレクチンおよびリガンド糖鎖の発現をウェスタンブロット法により解析した。我々のADモデルマウスの結果ではE-セレクチンの発現誘導が観察されているのでヒト剖検脳においてもE-セレクチンを重点的に検証した。また、セレクチンが認識するシアリルルイスX様糖鎖リガンドの発現も同様に解析した。（医療法人さわらび会福祉村病院で死亡し病理解剖を行った症例の中で、採取サンプルの研究目的の使用に承諾が得られた剖検試料を解析した。）ヒト剖検脳サンプルは以下の方法で調整した。１）凍結脳サンプルを Tris-buffered saline（TBS)に懸濁し超音波処理した。遠心後の上清を TBS 可溶画分として回収した。２）遠心後の沈殿物を 1%SDS に懸濁し超音波処理した。遠心後の上清をSDS可溶画分として回収した。３）遠心後の沈殿物を 98%ギ酸に懸濁しギ酸可溶画分として回収した。骨髄由来ミクログリア前駆細胞に細胞外スルファターゼSulf遺伝子をウィルスベクターの使用により導入し、ADモデルマウスに移植する試験の技術開発を行った。実験解析の実施はすべて本研究者の所属機関とし、徹底した当該機関の倫理審査の承認のもとで行った。医療法人さわらび会福祉村病院で死亡し病理解剖を行った症例の中で、採取サンプルの研究目的の使用に承諾が得られた剖検試料を解析した。

（倫理面への配慮）

研究実施に先立ち、各研究実施協力機関の倫理委員会による厳正中立な審査を受け、研究実施計画の承認を受ける。特にヒト試料を用いた研究実施に際しては人権の保護および個人情報の保護に最大限の注意を払うことを理解遵守し一層の徹底を図る。また、１）インフォームドコンセントの徹底、２）検体の使用及び保存についての中止請求を含む研究協力同意書の十分な説明、３）検体保存責任者を設置し当該者以外には連結不可能な匿名化を施したうえでのサンプル及びデータの保管、さらにスタンドアローンのコンピューターを用いたデータ処理、鍵のかかるキャビネット内へのデータ保管を行う。 本研究において遺伝子の抽出・保管および遺伝子発現の解析は行わず、遺伝情報に触れる事はない。

研究実施に先立ち、研究実施機関の倫理委員会による厳正中立な審査を受け、研

究実施計画の承認を受けた。本申請研究で実施するモデルマウス対象研究はすべて当該機関設置の遺伝子組換え生物実験安全委員会の審査を受け承認を得た。また、当該機関設置の実験動物委員会および動物実験倫理委員会の審査を受け承認を得た。本研究課題に参画する者は「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律（カルタヘナ法)」、「動物の愛護及び管理に関する法律」および「動物を用いる生物医学研究のための国際指導原則」の更なる理解を確認し遵守した。

C．研究結果

ヒト骨髄由来細胞株がモデルマウス脳血管内でローリングおよび接着を示す結果を得た。また、当該細胞でセレクチンリガンド糖鎖の発現が観察された。AD 嗅内皮質および海馬におけるセレクチンリガンド糖鎖の発現を検証したが困難であったため、リガンド糖鎖合成酵素の発現を検証した。AD 群においてセレクチンリガンド糖鎖合成酵素の一種が特異的に発現上昇を示した。リガンド糖鎖およびその合成酵素の AD 群における特異的発現上昇を確認した。神経毒性重合 Aβの除去を亢進させることが期待される細胞外スルファターゼ遺伝子をヒト細胞で発現し得るレンチウィルスベクターに組み込み、ウィルスを使用した遺伝子発現システムの開発に成功した。細胞外スルファターゼ遺伝子 DNA およびウィルス発現システムは本研究室で使用可能となった。

D．考察

AD 剖検脳におけるセレクチンリガンド糖鎖合成酵素の特異的発現上昇は AD モデルマウスで観察された骨髄由来ミクログリア細胞が AD 病態に伴って脳内へ移行するという現象がヒトでも起きている可能性をさらに強く示唆した。これらリガンド糖鎖合成酵素の発現はある種のサイトカインにより制御されている。この事から，サイトカインに注目したメカニズム解明の研究への発展も今後期待された。

E．結論

アルツハイマー病の病態に伴う骨髄由来ミクログリア細胞の脳内移行がヒト AD 脳でも起きている可能性が示された。神経毒性重合 Aβの除去を亢進させることが強く期待される細胞外スルファターゼ遺伝子を脳移行性細胞に恒常的に発現させ、血行性にアルツハイマー病態脳内へ送り込む細胞医薬開発の技術基盤を確立した。本ウィルス発現システムを今後の臨床応用研究で使用することが強く期待された。

F. 健康危険情報

該当なし。

G. 研究発表

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Annual meeting of Glycobiology 2011, Seattle, USA, Nov 11, 2011

H. 知的財産権の出願・登録状況（予定を含む。）
１．特許取得
なし

２．実用新案登録
なし

３．その他
なし

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hosono-Fukao, T., Ohtake-Niimi, S., Hoshino, H., Britschgi, M., Akatsu, H., Hossain, M. M., Nishitsuji, K., van Kuppevelt, T. H., Kimata, K., Michikawa, M. Wyss-Coray, T. and Uchimura, K.	Heparan sulfate subdomains that are degraded by Sulf accumulate in cerebral amyloid beta plaques of Alzheimer's disease: Evidence from mouse models and patients.	Am. J. Pathol.,	180	2056-2067	2012
Fujiwara M, Kobayashi M, Hoshino H, Uchimura K, Nakada T, Masumoto J, Sakai Y, Fukuda M, Nakayama J.	Long-form N-Acetylglucosamine-6-O-Sulfotransferase 1 in Human High Endothelial Venules.	J. Histochem. Cytochem.	60	397-407	2012

研究成果の刊行物・別刷

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Matrix Pathobiology

Heparan Sulfate Subdomains that are Degraded by Sulf Accumulate in Cerebral Amyloid ß 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 (Aβ). 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 Aβ 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 subdomains[7–9] 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 vasculature[9] 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 (Aβ). A great deal of biochemical and genetic evidence has indicated that aggregation and accumulation of Aβ 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 Aβ fibril formation and protection of the fibril against microglial phagocytosis.[16] It was reported that the aggregation state of Aβ 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-glial 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, ϕ1.4 nm, was from Nanoprobes (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 Choju Medical Institute of Fukushimura 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 μL (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 × *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 μL (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-μm 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-μm 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-μm 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 μg/mL), Cy2-conjugated polyclonal goat anti-rabbit IgG (3 μg/mL), and aminomethylcoumarin acetate-conjugated streptavidin (6.8 μg/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 μL of a reaction mixture containing 5 μmol HEPES, pH 7.5, 1 μmol $MgCl_2$, and enzymes at 37°C overnight. Recombinant human Sulf-1 (0.4 μg) and human Sulf-2 (0.4 μg) 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-μm thickness) were obtained from paraffin-embedded tissue blocks. After deparaffinization and rehydration, endogenous peroxidase activity was quenched with 3% H_2O_2 (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′-diaminobenzidinetetrahydrochloride) 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 μg/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 μg 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, pH8.0, 10 mmol/L $MgCl_2$ for 3 hours at 37°C. Subsequently, the samples were treated with actinase E (0.08 mg/mL) (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) overnight at 37°C. The supernatant was collected by centrifugation at 5000 × *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 μg was used for reverse transcription reaction in 100 μL of buffer with random hexamers, using Superscript II Reverse Transcriptase (Invitrogen). PCR was conducted in duplicate with 20-μL reaction volumes of SYBR Premix Ex TaqII (Takara Bio Inc. Shiga, Japan), 0.2 μmol/L of each primer and 2 μL 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′-GCAGATGGCCCTGAACAAGAA-3′ and 5′-GCACGTGCACAGGGTACACA-3′; for N-deacetylase/*N*-sulfotransferase 2 (*Ndst2*), 5′-TCATCCAGAAGTTCCTGGGTATCAC-3′ and 5′-AGACAGCGAGTCTTACCACCTTCAA-3′; for *Ndst3*, 5′-TCTGGTGTCAGCTGCTGGAAG-3′ and 5′-CACGTTGTGGTCGCGGTAGTAG-3′; for *Ndst4*, 5′-TTGTTCCCAAAGCCAAGATCATTAC-3′ and 5′-TCAGGGCAGCTGGATCTTCA-3′; for *Hs6st1*, 5′-CTGACTGGACCGAACTCACCAA-3′ and 5′-TCTCGCAGCAGGGTGATGTAGTAG-3′; for *Hs6st2*, 5′-AAACTTCAACTCAGGCGCCAAC-3′ and 5′-CTCCATTCACTCAAGTACCGTGACA-3′; for *Hs6st3*, 5′-GACTGGACCGAGCTCACCAA-3′ and 5′-CATGCTTCCATTCGCTCAGGTA-3′; for *Hs2st1*, 5′-GCAAGCACCTCGTTCACCAA-3′ and 5′-CATCTCGTTCCAGGTGGTTATGTTC-3′; for *Sulf1*, 5′-CCACATGGAGTTCACCAACGTC-3′ and 5′-TAGCCGTGGTCCGCAGTGTA-3′; for *Sulf2*, 5′-GAGTACCAGACAGCATGCGAACA-3′ and 5′-TTGGGCACCAGGTTGGAGA-3′; and for *Actb*, 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ and 5′-ATGGAGCCACCGATCCACA-3′.

Statistical Analysis

All data are presented as means ± 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.

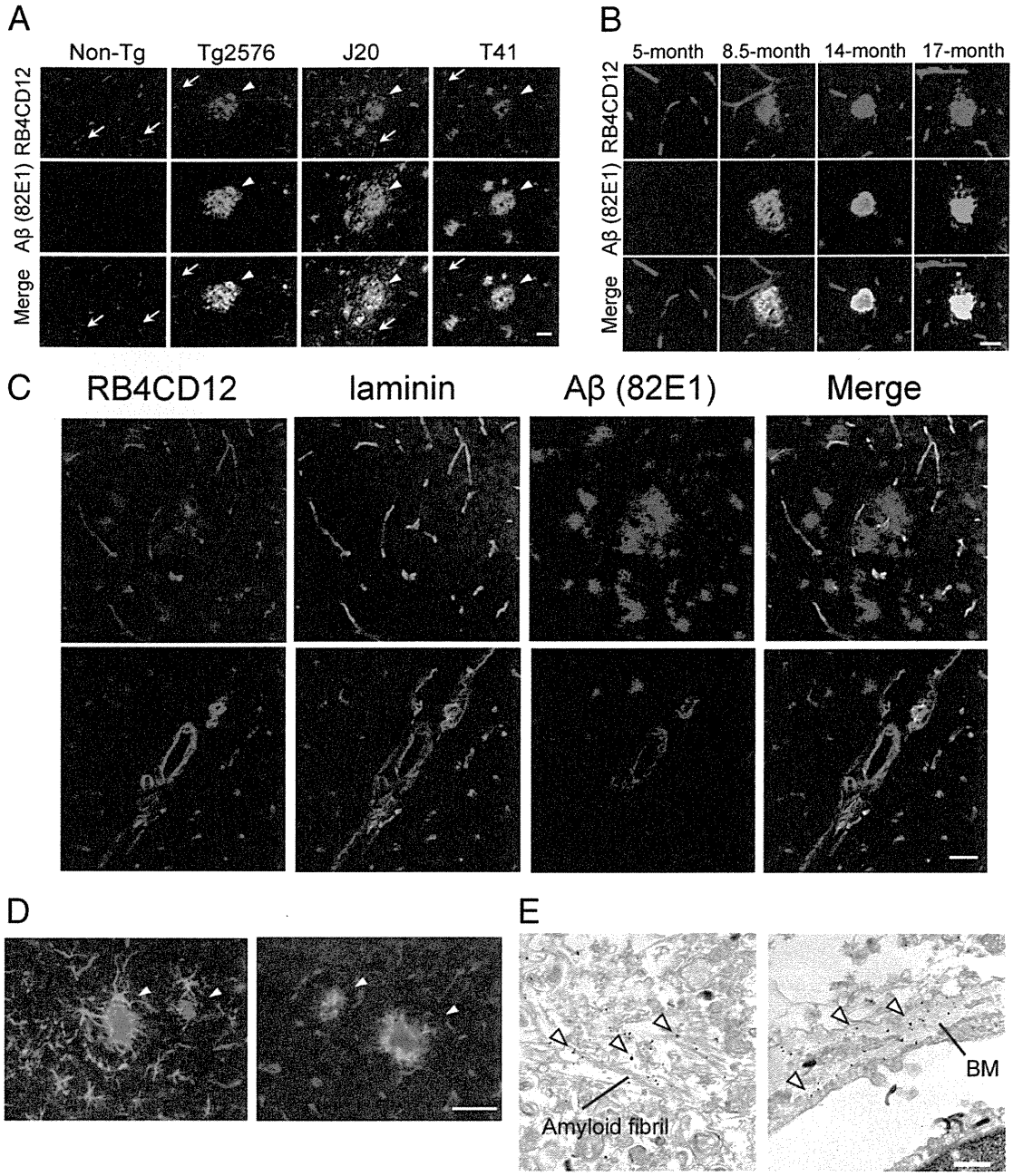

Figure 1. RB4CD12 anti-heparan sulfate epitope colocalizes with amyloid β plaques in the brain of Tg2576, J20, and T41 mice. **A:** Cryostat-cut brain sections of 18-month-old non-Tg and Tg2576, 23-month-old J20, and 12-month-old T41 mice were stained with RB4CD12 anti-HS antibody (red) and 82E1 anti-amyloid β (Aβ) antibody (green). Staining signals in vessels (**arrows**) and amyloid plaques (**arrowheads**) are shown. **B:** Expression of the RB4CD12 epitope and Aβ in aging Tg2576 brain. **C:** Expression of the RB4CD12 epitope, Aβ 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 μm. Scale bars (in **B** and **E**): 20 μm and 500 nm, respectively.

Results

Immunoreactivity of RB4CD12 Anti-Heparan Sulfate is Colocalized with Aβ 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 Aβ plaques in the cortex and hippocampus.[18] The localization of the RB4CD12 highly sulfated domains in Aβ 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 Aβ peptides, Aβ42 is dominant in J20 and T41 mouse brains, whereas Aβ40 is dominant in Tg2576 mouse. We examined brain sections of these model mice immunohistochemically. The RB4CD12 highly sulfated domains were colocalized with Aβ 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 Aβ plaques, we collected Tg2576 brains from 5-, 8.5-, 14- and 17-month-old mice. Aβ plaques were observed in 8.5-, 14- and 17-month-old Tg2576 brains. Cerebral Aβ deposition increases with age. RB4CD12 stained Aβ plaques at these ages (Figure 1B). Next, we investigated vasculature and non-vasculature RB4CD12 epitopes in aged Tg2576 brain by co-staining with antibodies against Aβ 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-Aβ staining signals in the cortex of Tg2576 mice (Figure 1C, upper panels). The RB4CD12 epitope was also observed in the vessel walls of Aβ-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 Aβ 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 Aβ 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 heparitinases. 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

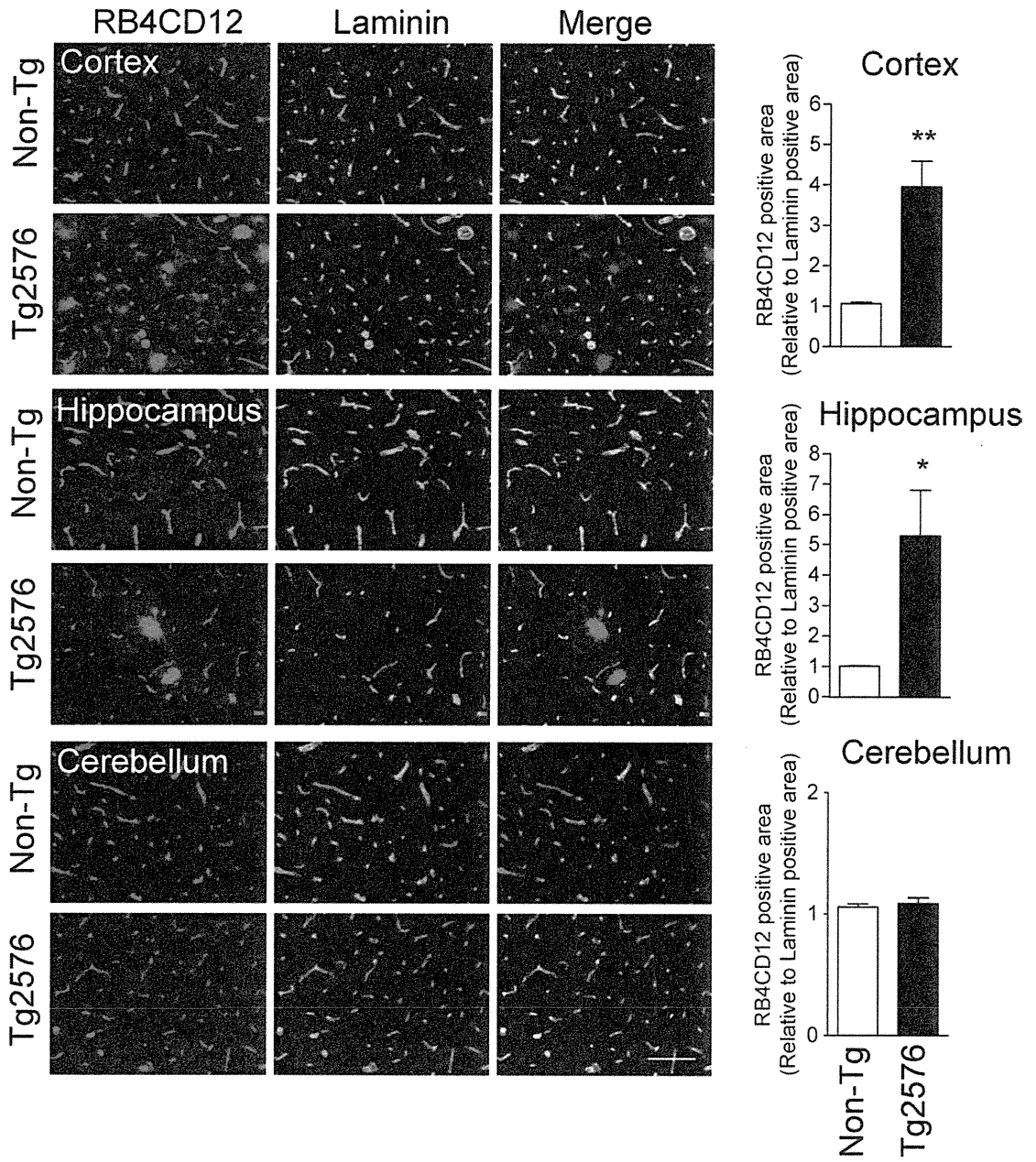

Figure 2. Quantification of the RB4CD12 epitope in nonvascular parenchyma in the brain of Tg2576 mice. Cryostat-cut brain sections of Tg2576 mice were stained with RB4CD12 (red) and anti-laminin (green) antibodies. Laminin is a marker for brain vessels. Nonvascular amyloid β plaques were stained with 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-Aβ staining signals that were colocalized with the RB4CD12 epitope were retained after Sulf treatment (Figure 5).

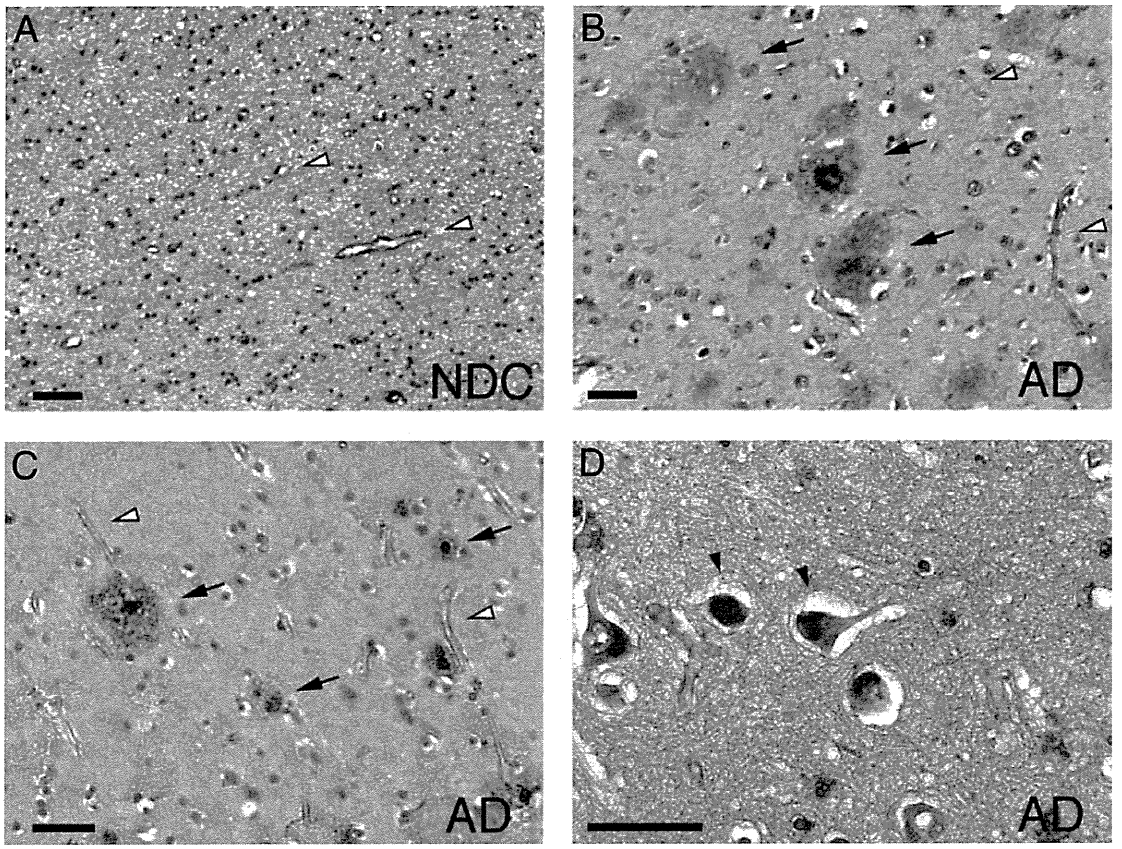

Figure 3. RB4CD12 epitope immunolocalizes in amyloid plaques in the brains of Alzheimer's disease patients. Immunoperoxidase staining for RB4CD12 (brown) in the entorhinal cortex (**A**, **B**) and hippocampus (**C**, **D**). **Open arrowheads** in **A**–**C** show vessel-staining signals in brains of 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 μm.

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 Aβ aggregates induce the structural and functional disruption of smooth muscle cells in the vasculature.[24] Results in aging brains of Tg2576 mice suggested that Aβ and the HS highly sulfated domains start accumulation at the same age. Aβ 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 Aβ.[34–36] *In vivo* fragmentation of heparan sulfate by heparanase overexpression could protect mice from amyloid protein A amyloidosis.[37] Importantly, the degree of sulfation is critical for enhancement of fibrillogenesis of Aβ.[35] Pathological effects of heparin in Aβ 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 Aβ 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 al[40] demonstrated that only a minimal number of Aβ 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 [-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

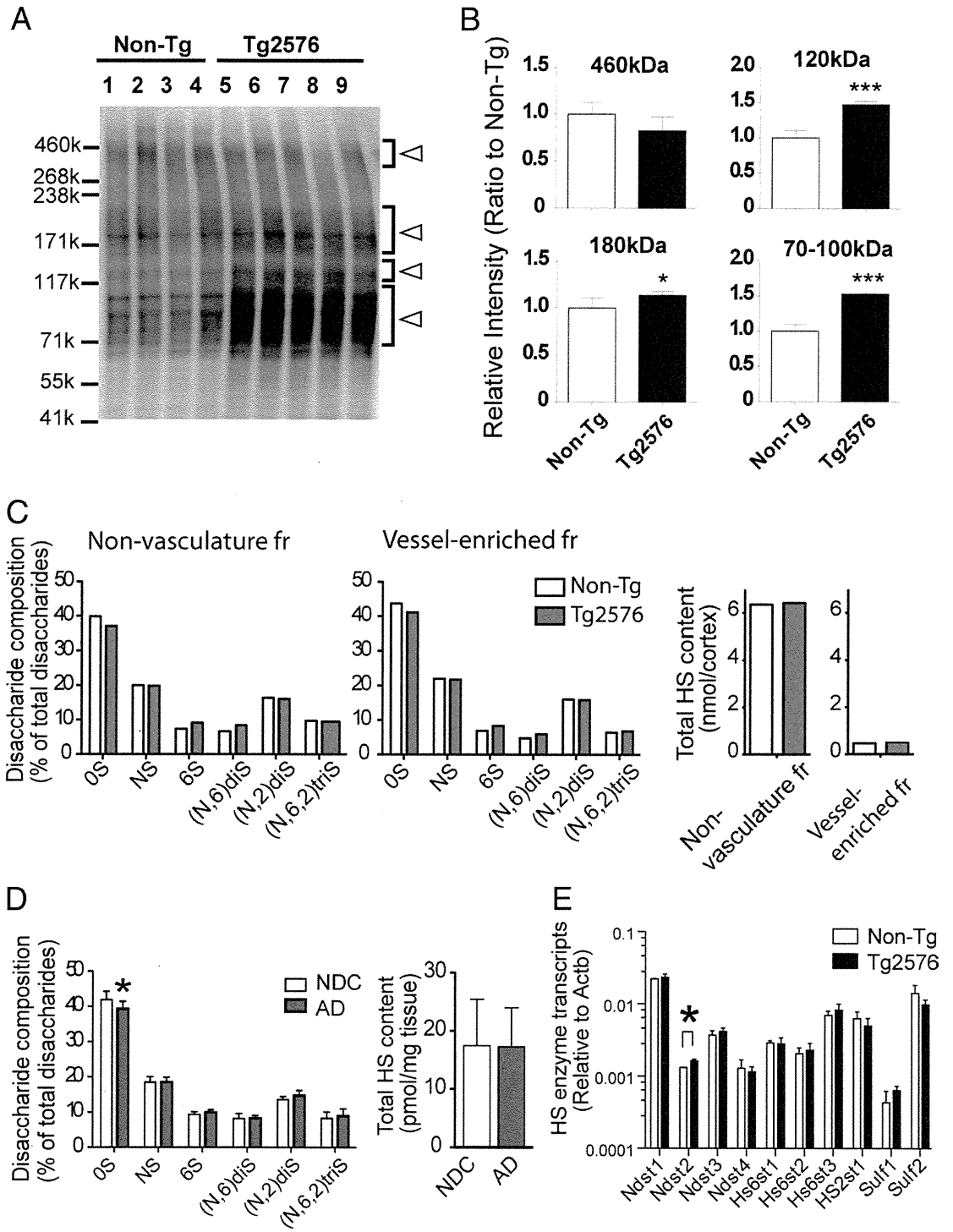

Figure 4. Immunoblotting analysis of the RB4CD12 epitope, disaccharide composition analysis of Heparan sulfate (HS) and quantitative real-time PCR analysis of HS enzymes in the brain of Tg2576 mice. **A:** Tris-buffered saline (TBS)-insoluble/1% SDS-soluble fractions were prepared from tissue homogenates 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 (0S), monosulfated (NS, 6S), disulfated ([N,6]diS, [N,2]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

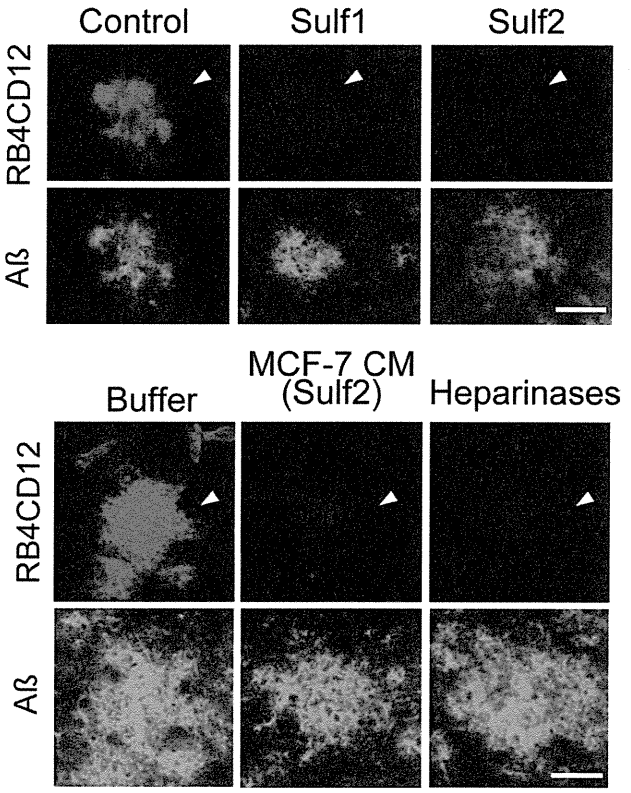

Figure 5. The RB4CD12 epitope in amyloid plaques of Tg2576 mouse brains is degraded *ex vivo* by Sulf-1, Sulf-2 and conditioned medium of Sulf-2-expressing cells. Cryostat-cut consecutive sections of 18-month-old Tg2576 mouse brains were incubated overnight with recombinant human Sulf-1 and Sulf-2 prepared 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-Aβ antibody (green). The data are representative of two independent experiments. **Arrowheads** indicate amyloid plaques. Scale bars: 20 μm.

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 Aβ 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 postmortem 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 al[52] 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 Aβ 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 Aβ 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 Aβ 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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