主論文の要旨

Reduced molecular size and altered disaccharide composition of cerebral chondroitin sulfate upon Alzheimer's pathogenesis in mice

（アルツハイマー病変に伴う脳コンドロイチン硫酸の分子量減少と二糖組成の変化：マウスモデルを用いた解析）

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Summary

Alzheimer's disease (AD) is a progressive disorder leading to cognitive impairment and neuronal loss. Cerebral extracellular accumulation and deposition of amyloid ß plaques is a pathological hallmark of AD. Chondroitin sulfate (CS) is an extracellular component abundant in the brain. CS is a sulfated glycosaminoglycan covalently attached to a core protein, forming chondroitin sulfate proteoglycan. The structure of CS is heterogeneous with sulfation modification and elongation of the chain. Various roles of CS in the brain are provided by its structural diversity. Increasing evidence has shown that CS promotes aggregation of amyloid ß peptides into higher-order species such as insoluble amyloid ß fibrils. Difficulties in a structural analysis of brain CS as well as its heterogeneity limit the study of potential roles of CS in AD pathology. Here we established a microanalysis method with reversed-phase ion-pair high performance liquid chromatography and found that CS in the brain of Tg2576 AD model mouse shows a lower molecular size and an increased ratio of the CS-B motif di-sulfated disaccharide. Our findings provide insights into the structural change of cerebral CS upon Alzheimer's pathogenesis.

Materials and Methods

Animals

Tg2576 mice bearing human amyloid precursor protein with Swedish (K670N, M671L) mutation were purchased from Taconic Biosciences (Hudson, NY). Animals were kept under controlled environmental conditions and with standard food and water supply. The Nagoya University Institutional Animal Care and Use Committee approved the animal studies.

Preparation and structural analysis of brain CS

Brain CS was isolated as described previously for heparan sulfate and keratan sulfate with slight modifications. A snap-frozen mouse cerebral cortex (25 mg), hippocampus (15 mg) and cerebellum (35 mg) were prepared from 20-month-old non-transgenic (n=5) and Tg2576 mice (n=7). The frozen tissues were suspended in 2 ml volume of 0.2N NaOH and incubated overnight at room temperature. The samples were treated with DNase I, RNase A (0.04 mg/ml each; Roche) and actinase E (0.04 mg/ml; Kaken Pharmaceutical, Tokyo, Japan) overnight at 37 °C. The samples were subjected to DEAE-Sepharose column chromatography. The purified CS was treated with chondroitinase ABC. Yielded CS disaccharides were separated. The disaccharide compositions of the CS were determined by reversed-phase ion-pair high performance liquid chromatography (HPLC) with post-column fluorescent labeling (Fig. 1).

Size exclusion chromatography

Purified CS from the cerebral cortex (100 mg) was obtained from 17-month-old non-transgenic (n=3) and Tg2576 mice (n=3) as descripted above. The samples were treated

with hyaluronidase. Superdex 200 16/60 column was used to separate the CS by a size fractionation. One-ml fractions were collected. The samples were digested with chondroitinase ABC. Post-column labeling reversed-phase ion-pair HPLC was performed to analyze the fractions. The level of CS content was determined by summing the amounts of all disaccharides detected in each sample.

Results

Total amount of CS is reduced in the cerebral cortex of Tg2576

Parenchymal amyloid plaque deposition starts to occur in the cerebral cortex and hippocampus of 11-13-month-old Tg2576 mice. The CS was purified from the brain parts and analyzed by reversed-phase ion-pair HPLC (Fig. 1). We found that the total amount of CS in the cortex of Tg2576 was reduced to 56 % of the non-transgenic (Non-Tg) level (Fig. 2).

CS molecular size is reduced in the cerebral cortex of Tg2576

We then asked if the molecular size of brain CS is altered with Alzheimer's pathology. To address this question, we extracted the CS from the cerebral cortex of 17-month-old Tg2576 and their Non-Tg littermates. The CS was subjected to size exclusion chromatography using the Superdex 200 column. The shift to a later value in retention time was observed in the peak of the Tg2576 CS (Fig. 3). Molecular mass of the peak position was 68 kDa for the CS extracted from Non-Tg mice, while that of the CS from Tg2576 mice was 58 kDa (Fig. 3).

Di-sulfated disaccharide components are increased in the CS of Tg2576 brain

We analyzed CS disaccharide composition in the cortex, hippocampus and cerebellum of 20-month-old Tg2576 and their Non-Tg littermates. In all tissues analyzed, we found that the 4S mono-sulfated disaccharide was a major CS component with 87 to 96 % of total disaccharides, and that the level of the 2S mono-sulfated disaccharide was under the detection level in both genotypes (Fig. 4). Percentage of DiS_E di-sulfated disaccharide is significantly upregulated 1.4- to 1.6-fold in cortex, hippocampus and cerebellum of Tg2576 mice as compared to that of Non-Tg (Fig. 4 and Table 1). In the Tg2576 cortex and hippocampus, where amyloid plaque deposition is abundant, percentage of DiS_B di-sulfated disaccharide is significantly upregulated 1.3- to 1.7-fold. These upregulations were not observed in the Tg2576 cerebellum, in which amyloid plaque deposition is absent (Fig. 4 and Table 1).

Discussion

Cortical areas abundant in CS proteoglycans are less susceptible to cytoskeletal changes in AD patients. It is likely that CS may have a protective effect on AD pathology. Reduction in the amount of the CS might cause the mice more affected by Aß-induced damages. Although mechanisms of down-regulation of the cortical CS in Tg2576 mice are

unknown, potential roles of the CS, especially in an ameliorative way, should be explored with administration of CS or genetic manipulation of the CS synthesis in the AD model mice. Recent progress revealed that low molecular weight CS is protective against Aß-induced neurotoxicity *in vivo*. Our finding that the molecular size of CS in Tg2576 was lower than that in Non-Tg might be a response in defense mechanism. Determining expression levels of the CS sulfotransferases and elucidating their acting mechanisms on cerebral CS will be forthcoming challenges.

Disaccharide constituents of CS from the cortex of AD and control patients were previously shown. The levels of 0S, 4S and 6S were determined. It was reported that none of those were changed in a group of AD. This finding is consistent with our present study with Tg2576 AD model mice, of which the level of either 0S, 4S or 6S disaccharide was unaltered. Our method is now capable of determining the levels of di-sulfated and tri-sulfated disaccharide components as well as the non- and mono-sulfated disaccharides. Taking this advantage, we did find that the DiS_B and DiS_E di-sulfated disaccharide components are upregulated in the Tg2576 brain. Since the upregulation of the DiS_B level was found in the cortex and hippocampus but not in cerebellum where amyloid plaques are absent, it is plausible that the increase in the DiS_B level may correlate with amyloid plaque formation and deposition. Increased level of the DiS_B in Tg2576 could be a result of responding neurotoxic Aß species and reducing their neurotoxicity in the cortex and hippocampus. The DiS_E di-sulfated disaccharide was upregulated in all three tissues. It could be possible that increase in the DiS_E di-sulfated disaccharide might associate with AD progression within the whole brain rather than amyloid-specific regions. The CS that contains the CS-E disaccharide structure, which is the saturated disaccharide form of the DiS_E, has shown to be a side chain covalently attached to appican, the proteoglycan form of the amyloid precursor protein. Upregulated levels of the DiS_E in the Tg2576 brain could be consequent on expression of the transgene product. CS is present as side-chains covalently bound to scaffold proteins in tissues. It is like that reduction in the total amount of CS would reflect to changes in the CS that are in a protein-bound form. Application of the assays described herein to identification and quantification of the core proteins should enhance our understanding of the potential roles served by the CS. Furthermore, by analyzing human postmortem AD brains with our established method, we would obtain some clues to clarify mechanisms of these upregulation and potential mitigating roles of CS in brains of AD patients upon Alzheimer's pathogenesis.

Conclusion

We found that total amount and average molecular size of the CS were significantly reduced in the cerebral cortex of Tg2576 mice. Moreover, in the cerebral cortex and hippocampus but not in the cerebellum, the component of DiS_B, the unsaturated form of the CS-B motif di-sulfated disaccharide, was upregulated in Tg2576 mice. These results clearly indicate that the CS structures are altered upon Alzheimer's pathogenesis.