Correlation of C4ST-1 and ChGn-2 expression with chondroitin sulfate chain elongation in atherosclerosis

VITA YANTI ANGGRAENI

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氏　　名	VITA YANTI ANGGRAENI
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【 学位論文題目 】

Correlation of C4ST-1 and ChGn-2 expression with chondroitin sulfate chain elongation in atherosclerosis(動脈硬化症におけるグリコサミノグリカン糖鎖伸長に関連する C4ST-1 と ChGn-2 の発現解析)

審 査 委 員

主 査　教 授　中村　俊一
教 授　秋田　穂束
教 授　伊藤　智雄

学位論文の内容要旨

Correlation of C4ST-1 and ChGn-2 expression with chondroitin sulfate chain elongation in atherosclerosis

動脈硬化症におけるグリコサミノグリカン糖鎖伸長に関連する C4ST-1 と ChGn-2 の発現解析

神戸大学大学院医学系研究科医科学専攻
循環器内科学
(指導教員：平田健一教授)
Vita Yanti Anggraeni

Key words: atherosclerosis, proteoglycans (PGs), glycosaminoglycans (GAG), chondroitin sulfate (CS), chondroitin 4-sulphotransferase-1 (C4ST-1), chondroitin *N*-acetylgalactosaminyltransferase-2 (ChGn-2)

The "response to retention" hypothesis of atherosclerosis was first outlined by Williams and Tabas in 1995 which proposed that subendothelial retention of atherogenic lipoproteins, such as low density lipoprotein (LDL) by extracellular matrix (ECM) molecules, particularly chondroitin sulfate (CS)/dermatan sulfate (DS) proteoglycans (PGs) is the critical initiating step in the atherogenesis. Although PGs are present in the normal arterial wall, they vary in their core proteins, sulfation pattern, and GAG chain length. These structural differences change dynamically during the progression of atherosclerosis.

Biglycan is a common type of CS/DS PG that is colocalized with apolipoprotein (Apo) B in early and advanced human atherosclerotic coronary arteries. Hyperelongated biglycan has been proposed as the critical factor for the development of atherosclerosis because it will promote the progression of atherosclerosis by enhancing the binding affinity to LDL. Hence, CS chain elongation has been proposed as a target for the prevention of atherosclerosis. The increase in chain length may be due to increases in the amount or activity of the enzymes that synthesize the CS chain. However, the molecular mechanism of CS chain elongation *in vivo* is not clear. Recent *in vitro* study performed by Izumikawa et. al. demonstrated that chondroitin 4-sulphotransferase-1 (C4ST-1) regulated the chain length and amount of CS in cooperation with chondroitin *N*-acetylgalactosaminyltransferase-2 (ChGn-2) in culture cells. However, the expression and role of *C4ST-1* and *ChGn-2* enzymes in atherosclerosis development *in vivo* have not been studied yet. Here we investigated the expression of *C4ST-1* and *ChGn-2* during atherosclerosis development *in vivo* and investigated whether their expression correlated with CS chain elongation.

We use low-density lipoprotein receptor knockout (LDLr KO) mice that were fed standard CRF-1 mouse chow (Charles River Laboratories International, Inc.) until 10–12 week of age and subsequently switched to F2HFD1 mouse chow with 1.25% cholesterol

to simulate a western diet (Oriental Yeast Co., Ltd., Japan) for 0, 2, 4, and 8 weeks to observe the development of atherosclerosis. Fresh frozen aortas from LDLr KO mice that were fed a western diet for 0, 2, 4, or 8 weeks were used to analyze *C4ST-1*, *C4ST-2*, *ChGn-1*, *ChGn-2*, *ChSy-1*, *ChPF* m RNA levels, disaccharide composition, and CS chain length. The heart (containing the aortic sinus) was either frozen in Tissue-Tek OCT (Sakura Finetek USA, Inc.) for quantification of atherosclerotic plaque area or processed for paraffin sectioning for immunohistochemistry experiments.

In the present study we found that disaccharide composition analysis from aortas of LDLr KO mice during atherosclerosis development, showed the increasing of total amount of CS comprised of ΔHexA-GalNAc (4S) approximately 74% and also DS as the atherosclerosis progressed. Our results also demonstrated that the synthesis of longer CS chains during the development of atherosclerosis is accompanied by increased expression of *C4ST-1* and *ChGn-2*. Furthermore, by immunostaining we showed that these enzymes colocalized with biglycan and apo B, which were already present at the initial stage of atherosclerosis. In contrast, their homologs, *C4ST-2* and *ChGn-1*, did not appear to be involved in CS chain elongation since their expression levels did not change significantly during the progression of atherosclerosis. We also observed that the mRNA expression levels of *ChSy-1* and *ChPF* increased significantly after the mice had consumed a western diet for 8 weeks. Based on the recent *in vitro* study performed by Izumikawa et. al, who demonstrated that *C4ST-1* and *ChGn-2* regulated the chain length and amount of CS through chondroitin polymerase consisting of *ChSy-1* and *ChPF in vitro*, our results suggested these 2 enzymes may be involved in the elongation of CS chains in the arterial wall during the progression of atherosclerosis.

The binding of atherogenic lipoproteins to arterial wall PGs is mediated by ionic interactions between the positively charged residues of apo B and negatively charged CS of PGs. Treatment of CS with chondroitinase ABC (ChABC) which selectively removes CS and DS chains from PGs, eliminated almost all of the apo B immunoreactivity in atherosclerotic lesions, it is likely that apo B no longer bound PGs because the CS chains were not present. We hypothesize that the residual apo B could not be removed by ChABC digestion because they were bound to PGs indirectly, via intermediate molecules, such as lipoprotein lipase. Nevertheless, our results suggested that the CS side chain is essential for apo B to bind CS/DS PGs, and therefore it has a critical role in the initiation and progression of atherosclerosis.

In conclusion, our results showed that *C4ST-1* and *ChGn-2* are involved in CS chain elongation during the development of atherosclerosis. However, we have not yet elucidated their mechanism of elongating CS chains. Currently, we are conducting *in vitro* and *in vivo* experiments with these enzymes by using siRNA-mediated knockdown and knockout mice, to determine their roles in the progression of atherosclerosis. Since *C4ST-1* and *ChGn-2* may be involved in the retention of atherogenic lipoproteins by mediating CS chain elongation, these enzymes may be a novel therapeutic targets to prevent the initiation and progression of atherosclerosis.

神戸大学大学院医学研究科（博士課程）

論文審査の結果の要旨			
受付番号	甲 第2182号	氏　名	Vita Yanti Anggraeni
論文題目 Title of Dissertation	動脈硬化症におけるグリコサミノグリカン糖鎖伸長に関連するC4ST-1とChGn-2の発現解析 Correlation of C4ST-1 and ChGn-2 expression with chondroitin sulfate chain elongation in atherosclerosis		
審査委員 Examiner	主　査 Chief Examiner 中村俊一 副　査 Vice-examiner 秋田穂束 副　査 Vice-examiner 伊藤智雄		

（要旨は1，000字～2，000字程度）

Subendothelial retention of lipoproteins by proteoglycans (PGs) is the initiating event in atherosclerosis. The elongation of chondroitin sulfate (CS) chains is associated with increased LDL binding and progression of atherosclerosis. Several glycosyltransferases and sulfotransferases are involved in the biosynthesis of CS chains. Recently, it has been shown that 2 Golgi enzymes, chondroitin 4-*O*-sulfotransferase-1 (C4ST-1) and chondroitin *N*-acetylgalactosaminyltransferase-2 (ChGn-2) in the presence of chondroitin polymerase consist of chondroitin polymerizing factor (ChPF) and any of these chondroitin synthase (ChSy) enzymes, *ChSy-1*, *ChSy-2*, or *ChSy-3*, play a critical role in CS chain elongation. However, the expression and role of *C4ST-1* and *ChGn-2* enzymes in atherosclerosis development *in vivo* have not been studied yet. In the present study, the candidate explored the expression of *C4ST-1* and *ChGn-2* during the progression of atherosclerosis *in vivo* and determined whether their expression correlated with CS chain elongation.

Low-density lipoprotein receptor knockout (LDLr KO) mice fed a western diet for 0, 2, 4, and 8 weeks were used to observe development of atherosclerosis. The extent of atherosclerotic lesions in the aortic root of mice was observed by oil red o staining. Dissacharide composition and CS chain length were analyzed by high performance liquid chromatography (HPLC). The binding of LDL and CS PG in this mouse model was confirmed by chondroitinase ABC (ChABC) digestion and apolipoprotein B (apo B) staining. Immunostaining was performed to assessed the expression of biglycan, apo B, *C4ST-1* and *ChGn-2* at the aortic sinus during atherosclerosis progression. The mRNA expression levels of biglycan, *C4ST-1*, *ChGn-2*, *C4ST-2*, *ChGn-1*, *ChSy-1* and *ChPF* were quantified using quantitative real-time polymerase chain reaction (qRT-PCR) technique.

Western diet treatment stimulated the development of atherosclerosis. The mice that were fed a western diet for 4 or 8 weeks had significantly more plaque areas than those that were fed the same diet for 0 or 2 weeks. Disaccharide composition analysis from aortas of LDLr KO mice during the development of atherosclerosis, showed the increasing of total amount of CS comprised of ΔHexA-GalNAc (4S) approximately 73% and also dermatan sulfate (DS) as the atherosclerosis progressed. The synthesis of longer CS chains during the

development of atherosclerosis was accompanied by increased expression of *C4ST-1* and *ChGn-2*. Treatment of CS with chondroitinase ABC (ChABC) which selectively removes CS and DS chains from PGs, eliminated almost all of the apo B immunoreactivity in atherosclerotic lesions, suggested that apo B no longer bound PGs because the CS chains were not present. These result demonstrated that CS side chain is essential for apo B to bind CS/DS PGs. Immunohistochemistry showed that *C4ST-1* and *ChGn-2* were colocalized with biglycan and apo B, which were already present at the initial stage of atherosclerosis and further increased as atherosclerosis progressed. In contrast, mRNA expression levels of their homologs, *C4ST-2* and *ChGn-1*, did not appear to be involved in CS chain elongation since their expression levels did not change significantly during the progression of atherosclerosis. The mRNA expression levels of *ChSy-1* and *ChPF* increased significantly after the mice had consumed a western diet for 8 weeks. Taken together, these results suggested that the CS side chain is essential for apo B to bind CS/DS PGs, therefore it has a critical role in the initiation and progression of atherosclerosis. *C4ST-1* and *ChGn-2* are involved in CS chain elongation during the development of atherosclerosis. *C4ST-1* and *ChGn-2* may be involved in the retention of atherogenic lipoproteins by mediating CS chain elongation. Therefore, *C4ST-1* and *ChGn-2* has a critical role in the initiation and progression of atherosclerosis through mediating the CS chain elongation.

The candidate has studied the correlation of *C4ST-1* and *ChGn-2* expression with CS chain elongation in atherosclerosis in vivo, and advanced the field of knowledge in the area of the roles and the mechanism of CS chain elongation during the progression of atherosclerosis. Thus, the candidate is recognized as having qualified for degree of Ph.D. (medicine).