学 位 論 文 の 要 旨

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学 位 論 文 名　Contribution of Cystatin C Gene Polymorphisms to Cerebral White Matter Lesions

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論 文 内 容 の 要 旨

INTRODUCTION

Cystatin C (CST3) is a 13 kDa protein that consists of 120 amino acids encoded by a 7.3-kb gene located on chromosome 20. CST3 is a major inhibitor of extracellular cysteine proteases in mammals. Extracellular matrix remodeling plays an important role in the development of arteriosclerosis as well as of atherosclerosis. In Binswanger's disease, arteriosclerotic stenosis of deep cerebral arteries is thought to be the major pathological cause, segmental loss of medial smooth muscle cells (SMCs) and proliferation of collagen fibrils in the adventitia have been observed in arteries. These imply that extracellular matrix remodeling plays an important role in the development of arteriosclerosis. In addition, it is reported that decreased levels of CST3 are associated with extensive destruction of elastin and collagen, while overexpression of cathepsins are linked to damage of the arterial wall. These observations suggest that reduction in the CST3 level could contribute to vascular remodeling processes that result from relative increases in protease activity.

CST3 levels have been found to be 5 times higher in the cerebrospinal fluid (CSF) than in the plasma. Given the potential pathophysiological role of CST3 in the vascular remodeling process described above, it may play an important role in cerebrovascular disorders that are caused as a result of arteriosclerosis, such as ischemic white matter lesions and lacunar infarction. So far, more than 100 single-nucleotide polymorphisms (SNPs) have been identified in the *CST3* gene, of which 2 have been suggested to have functional significance: 1) −82G/C (rs5030707), which is located in the 5′-untranslated region of the gene, and has been reported to affect promoter activity; 2) +148G/A (rs1064039), which is located in the coding region that elicits changes in

CST3 secretion. Furthermore, 3 SNPs, −82G/C, +4A/C (rs4994881), and +148G/A have been reported to be associated with serum CST3 levels. Therefore, the aim of this study was to investigate the effects of SNPs in the *CST3* gene contributing to cerebral white matter lesions. We focused on the 3 SNPs described above because of their putative functional significance.

MATERIALS AND METHODS

From December 2000 to October 2007, a total of 2676 Japanese subjects voluntarily participated in health checkups at Shimane University Hospital, Japan. Of the 2676 participants, 3 *CST3* gene polymorphisms were genotyped in 92 cases with severe deep white matter hyperintensity (DWMH) and 184 were randomly selected age- and sex-matched controls without any signs of DWMH. Genotypes of the *CST3* gene were determined by PCR-restriction fragment length polymorphism analysis. The genetic effects of these polymorphisms on DWMH and plasma CST3 levels were examined. In order to investigate the functional role of the +148 G/A polymorphism, the 2 allelic forms of the CST3 expression vector were transfected into CCF-STTG1 astrocytoma cells. The expression level of CST3 mRNA was analyzed by quantitative RT-PCR. Intracellular and secreted levels of CST3 in the cell culture were quantified by Western blot and ELISA, respectively.

RESULTS AND DISCUSSION

For all 3 SNPs, the frequencies of the minor allele and minor allele-containing genotypes were greater in the DWMH-positive subjects. The pair-wise linkage disequilibrium test showed that the 3 SNPs were in a significant linkage disequilibrium. Based on this result, haplotype analysis was performed in order to identify the 2 major haplotypes in this population. The frequency of −82C/+4C/+148A was higher in DWMH-positive subjects than DWMH-negative controls. Logistic regression analysis indicated that carriers of this haplotype were 2.46 times more likely to have DWMH compared to non-carriers. This haplotype was also associated with lower plasma CST3 levels ($p = 0.01$).

Although no significant differences were observed between the mRNA levels of cells transfected with the 2 vectors having different alleles, we found that the intracellular CST3 protein level was approximately 3 times higher in cells transfected with the +148A allele than those transfected with the +148G allele ($p < 0.01$). We then measured the amount of CST3 protein secreted into the conditioned culture media. The CST3 protein level was significantly lower in cell cultures transfected with +148A than those transfected with +148G.

In this study, we first reported that a minor haplotype (−82C/+4C/+148A) of the *CST3* gene was associated with a severe form of DWMH. After adjusting for eGFR in the multivariate analysis, we found that the same haplotype was associated with low plasma CST3 levels, which

suggests that CST3 plays a complex role in the pathogenesis of small-vessel cerebral diseases.

In general, high serum CST3 levels are believed to be associated with small-vessel cerebral diseases. Although the results of the present study seem to be inconsistent with previous reports, several studies have suggested that rather than circulating protein levels, imbalance between proteases and inhibitors in the local environment, such as the arterial wall, determines their net effects on the cardiovascular system. In fact, a high plasma CST3 level indicates a high risk for cardiovascular events, whereas reduced expression of *CST3* in the arterial wall of rodents and humans has an important role in the formation of aneurysms and atheromatous plaques. Thus, there is a discrepancy between CST3 levels in systemic circulation and local environments, such as the arterial wall. A possible explanation for this discrepancy is that the serum CST3 level is merely an indicator of DWMH and has little direct or causal effects on the pathogenesis of small-vessel cerebral diseases. A major determinant of the serum (plasma) CST3 levels is the GFR. In fact, there is no convincing evidence, which shows that the serum CST3 level directly increases the risk for cardiovascular diseases without contribution from reduced renal function or hypertension.

The present *in vitro* study indicates that +148 G/A is a functional SNP that affects extra and intracellular CST3 levels. On the other hand, the association analysis showed that the odds ratio of the haplotype containing +148A was greater than the +148A allele alone (4.01 vs. 1.67, respectively). The results of these biological and genetic studies seem to be inconsistent with each other. In order to interpret this discrepancy, it may be likely that the genetic effects of the A allele are recessive. According to the *in vitro* study, the A allele imposed a "loss-of-function" effect on the extracellular CST3 level, which is consistent with the observations of the association analysis.

Conversion of +148G to A leads to the substitution of threonine for alanine in the signal peptide of CST3, which may alter its hydrophobicity. The signal sequence hydrophobicity profile is known to be important in signal recognition processes and alternation of its hydrophobicity may modify the secretory processing pathway of CST3. According to our *in vitro* study, in addition to decreases in CST3 levels in the local environment, intracellular accumulation of CST3 could also exacerbate arteriosclerosis. Intracellular build-up and microvascular degeneration could also have resulted from the +148 G/A SNP in the signaling peptide.

CONCLUSION

This study indicates that SNPs in the *CST3* gene confer a risk for developing ischemic white matter lesions, possibly through altered secretion of CST3. Further studies on the molecular mechanisms of CST3 secretion and its effect on arteriosclerosis are warranted.