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Metabolic Syndrome Is Associated With Silent Ischemic Brain Lesions

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Background and Purpose—Metabolic syndrome (MetS) is a recognized risk factor for stroke, but it is unclear whether MetS is also related to subclinical ischemic lesions. We examined the association of MetS with the prevalence of silent brain infarction, periventricular hyperintensity, and subcortical white matter lesions in healthy adults.

Methods—We conducted a cross-sectional study in 1151 Japanese healthy subjects. Three types of silent lesions were assessed by MRI scans. MetS was diagnosed using the criteria by the National Cholesterol Education Adult Treatment Panel III.

Results—After adjusting for age and other factors, MetS was significantly associated with silent brain infarction, periventricular hyperintensity and subcortical white matter lesions. Among the MetS components, elevated blood pressure was commonly associated with all types of lesions. Dyslipidemia and elevated fasting glucose levels were associated with subcortical white matter lesions and periventricular hyperintensities, respectively. Positive trends were observed between the number of MetS components and prevalence of silent lesions.

Conclusions—MetS is associated with the prevalence of silent lesions independent of other risk factors. The clustering of MetS components tends to increase the prevalence of silent lesions. **(*Stroke*. 2008;39:1607-1609.)**

Key Words: metabolic syndrome ■ silent brain infarction ■ periventricular hyperintensity ■ subcortical white matter lesions

Prospective population-based cohort studies have demonstrated that metabolic syndrome (MetS) is a potent risk factor for stroke.[1,2] Very few studies have investigated the influence of MetS on silent ischemic brain lesions,[3,4] although they are regarded as warning signs for future stroke and cognitive deterioration.[5] In this study we investigated the association of MetS and 3 types of subclinical ischemic lesions: silent brain infarction (SBI), periventricular hyperintensity (PVH), and subcortical white matter lesions (SWMLs).

Materials and Methods

Subjects

We studied 1151 healthy persons (44 to 87 years) selected from 1518 Japanese adults, who voluntarily visited the Shimane Institute of Health Science for health screening. The screening system included medical and neurological examination, head MRI scans, and blood tests. The selection criteria were as follows: informed consent to this study, no history of psychiatric or neurological diseases including transient ischemic attack, no neurological abnormalities, and no missing data for complete analysis. Demographic data are shown in Table 1. The study was approved by the institutional ethics committee.

Criteria of Metabolic Syndrome

MetS was diagnosed based on the criteria from the National Cholesterol Education Program Adult Treatment Panel III,[6] modified for the Japanese people.[7] Although waist circumference is the preferred measure of central obesity in MetS diagnosis, it was not measured at the time of data acquisition. For this reason, we used body mass index (BMI) as a substitute for waist circumference. The good correlation between them was obtained in a separate study.[8] Central obesity was defined as BMI ≥25.

Magnetic Resonance Imaging

Head MRIs were obtained using conventional pulse sequences for T2-weighted image, T1-weighted image, and fluid-attenuated inversion recovery (FLAIR) image in the transverse plane with a slice thickness of 7 mm by a 1.5-Tesla MRI (Symphony, Siemens).

Silent Brain Lesions

Brain infarction was defined as a focal hyperintensity lesion 3 mm or large in diameter in the T2-weighted image corresponding to a hypointensity lesion in the T1-weighted image. PVH and SWMLs were evaluated separately based on their distinct subcortical distributions. PVH was graded on a scale of 0 to 4 as described elsewhere.[9] SWMLs were graded on a scale of 0 to 3 according to the Fazekas' grading scheme.[10] We defined grades 0 to 2 PVH as PVH−, grades 3 to 4 PVH as PVH+, grades 0 to 1 SWML as SWML−, and grades 2 to 3 SWML as SWML+.

Statistical Analysis

We used Student t test, the Mann–Whitney U test, or χ^2 test in the group comparison. Logistic regression models were used to determine the association between silent lesions and risk factors or MetS

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Table 1. Demographic Data and Prevalence of MetS Components and Silent Brain Lesions

	Non-MetS (n=1030)	MetS (n=121)
Sex, male/female	519/511	96/25*
Age, y	62.6±6.5	62.7±7.9
Education, y	12.3±2.6	12.5±2.7
Smoking, %	33.4	54.5*
Alcohol habit, %	14.0	26.4†
Increased BMI, %	14.6	100*
Elevated blood pressure, %	40.2	91.0*
Dyslipidemia, %	21.7	62.3*
Only TG >150 mg/dL, %	19.0	50.0*
Only HDL <40 mg/dL, %	1.2	1.6
Both above, %	1.5	10.7*
Elevated fasting glucose, %	22.0	63.1*
SBI+, %	11.6	27.9*
PVH+, %	4.0	8.2*
SWMLs+, %	15.1	25.4*

TG indicates triglycerides; HDL, high-density lipoproteins. Definitions of smoker and alcohol habit were described elsewhere.[6]

*$P<0.0001$, †$P=0.0003$.

components. The trend analysis was performed for the association between the number of MetS components and silent lesions by assigning median values for the odds ratio for each category. $P<0.05$ was considered significant.

Results

MetS was more prevalent in men compared to women, and was associated with higher rates of smoking and alcohol consumption. Univariate logistic analyses revealed significant associations between MetS and SBI, PVH, or SWMLs (Table 2). Increased age, male sex, and smoking were significantly associated with SBI, whereas age was the only significant risk factor for PVH and SWMLs. Multivariate logistic analyses revealed that MetS was an independent risk factor for all 3 types of silent lesions.

Table 3 shows the effects of each MetS component on silent lesions. Multivariate logistic analyses revealed that increased BMIs, elevated blood pressure, and elevated fasting glucose were independent risk factors for SBI, elevated blood pressure and elevated fasting glucose for PVH, and elevated blood pressure and dyslipidemia for SWMLs.

The association between the number of MetS components and silent lesions are shown in Table 3. The prevalence of silent lesions was positively associated with the number of MetS components ($P=0.008$ for SBI and $P<0.1$ for PVH and SWMLs). These results did not change after adjusting for sex, age, or smoking habits. The inclusion of interactive variables across MetS components in the regression model showed no singificant effects on the prevalence of silent lesions.

Discussion

We found that MetS was associated with 3 major silent ischemic brain lesions. Although age was most strongly associated with all silent lesions, MetS was still an independent risk factor after the adjustment of age. This finding is important because PVH and SWMLs are considered risk factors for future cognitive impairment.[5] Our study basically confirmed recent studies[3,4] but showed that only marked PVH and SWML were associated with MetS. Age and hypertension are well established risk factors for PVH and SWMLs, but the histological changes underlying mild PVH are not always caused by ischemia.[11]

It is unclear how MetS is related to the pathology of small-artery disease, which is a major underlying pathology in silent lesions. It was reported that MetS contributed to both atherothrombotic and lacunar infarctions.[12] Various metabolic disturbances may promote pathological changes in the arteries, which usually begin in larger extracerebral arteries and then spread to smaller, distal, intracerebral arteries.[1]

Table 2. Association of MetS and Other Demographic Factors With SBI, PVH, and SWMLs

	SBI		PVH		SWML	
	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Univariate						
MetS (yes)	2.87 (1.84–4.47)	<0.0001	2.23 (1.09–4.58)	0.03	1.99 (1.28–3.10)	0.002
Age (per 1 year)	1.07 (1.04–1.10)	<0.0001	1.09 (1.05–1.14)	0.001	1.12 (1.09–1.14)	<0.0001
Sex (male)	2.22 (1.54–3.20)	<0.0001	1.11 (0.63–2.00)	0.71	0.96 (0.70–1.31)	0.77
Education (per 1 year)	1.04 (0.97–1.12)	0.26	1.02 (0.92–1.14)	0.71	0.95 (0.89–1.01)	0.10
Smoking (no)	0.57 (0.40–0.80)	0.001	0.69 (0.39–1.23)	0.21	0.98 (0.70–1.36)	0.89
Alcohol (no)	1.00 (0.98–1.02)	0.77	1.00 (0.92–1.08)	0.90	0.99 (0.92–1.07)	0.84
Multivariate						
MetS (yes)	2.43 (1.53–3.87)	0.0002	2.13 (1.02–4.44)	0.04	2.00 (1.25–3.21)	0.004
Age (per 1 year)	1.07 (1.04–1.10)	<0.0001	1.09 (1.04–1.13)	<0.0001	1.11 (1.09–1.14)	<0.0001
Sex (male)	1.85 (1.15–2.98)	0.01	...	...	...	...
Education (per 1 year)	...	...	...	...	...	...
Smoking (no)	0.87 (0.55–1.34)	0.54	...	...	...	...
Alcohol (no)	...	...	...	...	...	...

OR indicates odds ratio.

Table 3. Association of MetS Components With SBI, PVH, and SWMLs

	SBI		PVH		SWML	
	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Univariate						
Increased BMI	1.91 (1.32–2.75)	0.0005	1.27 (0.68–2.39)	0.46	1.31 (0.91–1.87)	0.14
Elevated blood pressure	1.77 (1.25–2.50)	0.001	2.40 (1.32–4.37)	0.004	1.54 (1.12–2.12)	0.008
Dyslipidemia	1.56 (1.09–2.23)	0.02	1.22 (0.67–2.25)	0.52	1.48 (1.06–2.07)	0.02
Elevated fasting glucose	2.10 (1.47–2.99)	<0.0001	2.72 (1.54–4.82)	0.0006	1.29 (0.91–1.82)	0.15
Multivariate						
Increased BMI	1.55 (1.05–2.27)	0.03	...	...	...	...
Elevated blood pressure	1.54 (1.08–2.20)	0.02	2.22 (1.22–4.05)	0.01	1.48 (1.07–2.03)	0.02
Dyslipidemia	1.24 (0.85–1.81)	0.27	...	...	1.40 (0.99–1.96)	0.05
Elevated fasting glucose	1.86 (1.29–2.67)	0.0008	2.53 (1.42–4.50)	0.002	...	...
No. of MetS components						
0	1.0	...	1.0	...	1.0	...
1	1.49 (0.93–2.46)	0.12	1.75 (0.74–4.15)	0.20	1.04 (0.68–1.59)	0.86
2	2.00 (1.19–3.37)	0.009	2.14 (0.88–5.25)	0.09	1.45 (0.93–2.26)	0.09
3	4.03 (2.28–7.14)	<0.0001	2.66 (0.94–7.50)	0.06	1.62 (0.93–2.81)	0.08
4	4.71 (2.11–10.5)	0.0002	7.76 (2.53–23.8)	0.0003	3.40 (1.62–7.12)	0.0012
P for trend	0.008		0.07		0.06	

OR indicates odds ratio.

The limitation of this study includes bias in subject selection due to nonrandomized design. Furthermore, longitudinal studies are obviously needed in the future for leading more general conclusions.

In conclusion, MetS was significantly associated with all 3 types of silent lesions after adjusting for age and other factors. The positive trend between MetS components and silent lesions could be used as a diagnostic tool to predict and prevent future stroke.

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Disclosures

None.

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Umegae N, Nagai A, Terashima M, Watanabe T, Shimode K, Kobayashi S, Masuda J, Kim SU, Yamaguchi S. Cystatin C expression in ischemic white matter lesions.
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Objectives – To study the involvement of cystatin C in the progression of ischemic white matter lesions (WMLs). *Materials and methods* – Cystatin C levels in the cerebrospinal fluid (CSF) of patients with cerebrovascular disease, and also in primary and established human neural cell cultures were investigated. For pathologic analysis, cystatin C immunoreactivity was investigated in the white matter of patients with severe WMLs, mild WMLs or controls. *Results* – Cystatin C levels in the CSF of patients with Fazekas WML grade 3 [14 with hypertension; W/HT(+) and nine without hypertension; W/HT(−)] were lower than those in 38 patients with grade 0–1 ($P = 0.0022$ and $P < 0.0001$ respectively). Immunohistochemical study showed that the cystatin C immunoreactivity was found in astrocytes, and the number of astrocytes in the white matter in the severe WML group was decreased when compared with that in controls ($P = 0.0027$) and in the mild WML group ($P = 0.0024$). In human neural cell cultures, treatments with thrombin, matrix metalloproteinases and interleukin 1β increased the expression of cystatin C mRNA in human astrocytes and hybrid neurons, but an enzyme-linked immunosorbent assay revealed that only thrombin significantly increased the production and secretion of cystatin C in astrocytes. *Conclusions* – These results suggest that low levels of CSF cystatin C in ischemic WMLs might be due to the decreased number of astrocytes that secrete cystatin C in response to the stimuli of proteases and inflammatory cytokines.

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Introduction

Cystatin C is an inhibitor of cysteine proteases, secreted from various cell types (1) and involved in the regulation of local inflammation (2), tumor invasion and metastasis (3). Its concentration in the cerebrospinal fluid (CSF) is 5.5-fold of that in serum and is a major cysteine protease inhibitor in the CSF (4).

Among the human neurological diseases, hereditary cerebral hemorrhage with amyloidosis in Iceland (HCHWA-I) is the only disease in which involvement of cystatin C is well established. In HCHWA-I, a truncated form of cystatin C, with a deletion of the first 10 amino-terminal residues, is deposited as amyloid fibrils in cerebral microvessels and most patients die from recurrent brain hemorrhage before 40 years of age (5). In these patients, there is an amino acid substitution at position 68 in cystatin C (glutamine for leucine) and the CSF level of cystatin C is one-third of reference groups (6). In previous studies, we have demonstrated that cystatin C levels in the CSF were decreased in patients with sporadic cerebral amyloid angiopathy (CAA) (7), of whom Binswanger type of progressive white matter change existed (8).

Growing evidence shows that CSF cystatin C levels are one of the indicators for the evaluation of the condition of cysteine protease system in various neurologic diseases. In inflammatory neurological diseases and leptomeningeal tumor metastasis, cystatin C may regulate their activity in the CSF (9, 10). However, the contribution of cystatin C to

the pathology of various diseases, including cerebrovascular diseases (CVDs) and the development of cerebral white matter lesions (WMLs), remains poorly understood. To clarify the role of cystatin C in the progression process of ischemic WMLs, cystatin C levels in the CSF were measured in patients with cerebral ischemia, and the expression of cystatin C was immunohistochemically determined in autopsied brain tissues. In addition, the expression and secretion of cystatin C were analyzed in the primary and established human neuronal cell culture systems.

Materials and methods

Patients and samples

Twenty-three patients with WMLs grade 3 were selected from the patients diagnosed with chronic ischemic CVD with lacunar infarctions at Shimane University Hospital between July 1993 and December 2004, of whom 14 patients were with hypertension [W/HT(+); mean age ± SD, 75.1 ± 5.2 years] and nine patients were without hypertension [W/HT(−); 75.9 ± 10.3 years]. Thirty-eight patients with WMLs grade 0 or 1 (control; 71.4 ± 11.6 years) were treated as control.

The magnetic resonance imaging (MRI) examination was performed on a 1.5-T MRI (Signa Horizon Cvi 1.5 T; GE, Fairfield, CT, USA). Brain infarction was defined as a focal hyperintense lesion > 3 mm in diameter on T1-weighted image (TR, 2000 ms; TE, 24 ms). Photon density-weighted or fluid-attenuated inversion recovery (FLAIR) images were used to distinguish infarcts from dilated perivascular spaces. WMLs were coded using the Fazekas score (11) as grade 0–3, where grade 0 = absent; grade 1 = punctate; grade 2 = beginning to be confluent and grade 3 = confluent.

All samples and autopsies used in this study were obtained under the informed consent of Shimane University School of Medicine. The CSF samples of patients in admission were centrifuged to remove the cell fractions and kept frozen at −70°C until use. The post-mortem delay of the autopsy ranged from 2.0 to 9.0 h (mean 4.2 h). The brains were fixed in 10% formalin and brain slices were cut coronally and embedded in paraffin wax.

For the immunohistochemical analysis, we examined a total of 10 brains (Table 1), which included three brains of ischemic CVD with severe white matter lesions (sWMLs), three brains of ischemic CVD with no or mild WMLs (mWML) and four controls with no significant WMLs in three patients with Parkinson's disease and a patient with cancer. In the three brains with severe WMLs, none showed the pathological features of Alzheimer's disease (AD) (12) or atherosclerosis of large vessels. A patient was immunohistochemically confirmed as CAA. All hypertensive patients were treated with antihypertensive drugs.

Table 1 Patient profile for immunopathological study

Group	Age	Sex	CSFcysC (pg/ml)	Diagnosis
Severe white matter lesion (sWML)	81	M	450	Binswanger
	70	M	670	Binswanger, HT
	84	M	310	CAA
Mild white matter lesion (mWML)	74	M	1070	HT, DM
	74	M	700	HT
	61	M	1900	HT
Control (C)	74	M	1050	PD
	81	M	1200	Prostate cancer
	79	M	600	PD
	71	F	940	PD

CAA, cerebral amyloid angiopathy; HT, hypertension; DM, diabetes mellitus; PD, Parkinson's disease.

Cystatin C levels

Cystatin C levels in the CSF were measured using an established method of an enzyme-linked immunosorbent assay (ELISA) (7). In brief, monoclonal mouse anti-cystatin C antibody, which was provided by Dr Anders Grubb, was coated on a 96-well microplate (Nunc, Roskilde, Denmark). Then, 100 µl of samples or cystatin C standard (Ohtsuka Inc., Tokushima, Japan) was placed in the wells at room temperature for 2 h, and polyclonal rabbit anti-cystatin C antibody (Dako, Copenhagen, Denmark) was added to each well. One hour after adding peroxidase-labeled goat anti-rabbit IgG antibody (Zymed, San Francisco, CA, USA), each sample was reacted with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt. The absorbance of the reaction product was measured at 417 nm with a microplate reader.

Cystatin C levels in cell fractions or culture supernatant were also determined by the ELISA. Cultures of astrocytes or A1 human hybrid neurons grown in six-well culture plates were stimulated by 5 U/ml of thrombin, 0.5 µg/ml of matrix metalloproteinase-2 (MMP-2), 10 ng/ml of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) or γ-interferon (IFN-γ) for 48 h; culture supernatants were collected and stored at −80°C. Cystatin C levels in assays were corrected by the protein amounts of the cell lysate contained in each culture. Protein contents of the cultured cells were

determined according to the method of Lowry et al. using bovine serum albumin as the standard (13).

Immunohistochemistry

Routine histological assessment was carried out with hematoxylin and eosin, and Klüver–Barrera and Bielschowsky staining. An immunohistochemical analysis was performed on 8-μm-thick sections from the frontal and temporal cortices using the ABC method (ABC kit; Vector, Burlingame, CA, USA). The following primary antibodies were used (Table 1): anti-glial fibrillary acidic protein (GFAP, rabbit; DAKO) for the detection of astrocytes, anti-KP-1 antibody for microglia (mouse monoclonal; DAKO), anti-galactocerebroside antibody for oligodendrocytes (mouse monoclonal; Chemicon, CA, USA) and anti-cystatin C antibody (rabbit; Upstate Biotechnology, New York, NY, USA). For double-labeling immunohistochemistry for GFAP, KP-1 or cystatin C, the first staining cycle was colorized for 10 min with a solution of 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB), 0.6% nickel ammonium sulfate and 0.00005% H_2O_2 in 50 mM Tris/HCl buffer. After the sections were immersed in 0.1 M glycine–HCl solution (pH 2.2) for 2 h to quench the primary antibody, the second cycle was processed and colorized for 5 min in 0.02% DAB and 0.1% H_2O_2 in 50 mM Tris/HCl buffer.

Cell cultures

Four primary culture series were prepared from embryonic human brains of 12–15 weeks' gestation as described previously (14). The use of embryonic tissue samples was approved by the Ethics Committee of the University of British Columbia Faculty of Medicine. Brains were dissected into small blocks and incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin and 40 μg/ml DNAse I for 30 min at 37°C. Dissociated cells were suspended in Dulbecco's modified Eagle's medium supplemented with 5% horse serum, 5% fetal bovine serum, 5 mg/ml glucose, 20 μg/ml gentamicin and 2.5 μg/ml amphotericin B (feeding medium), plated at a density of 10^6 cells/ml in T75 culture flasks at 37°C in an incubator with 5% CO_2/95% air atmosphere. After 2–4 weeks, microglia floating in the medium were plated on coverslips or dishes at appropriate densities. For the isolation of neuron-enriched populations, small neurons on top of the basal astrocyte layer were detached by mechanical shaking on an orbital shaker at 250 rpm overnight and collected. To enrich with neuronal population, cultures were exposed to 0.5 mM each of 5′-fluoro-2′-deoxyuridine (FrdU; Sigma, St Louis, MO, USA) and uridine (Sigma) for 24 h on days 4 and 7 in culture. After neurons and oligodendrocytes were removed, astrocyte-enriched cell preparations were obtained.

Cultures of human oligodendrocytes from adult brains were established using an enzyme digestion Percoll density gradient method as previously described (15). Brain tissue was minced, dissociated through a 100-μm nylon mesh, mixed with Percoll (Pharmacia Biotech, Uppsala, Sweden) and then centrifuged in a 30% Percoll gradient. A layer containing oligodendrocytes was collected, washed and suspended in feeding medium. Twenty-four to 48 h after plating, floating oligodendrocytes were collected and plated.

To confirm the purity of cell preparation, each cell type-enriched culture was processed for immunolabeling with antibodies against cell type-specific markers: anti-β-tubulin isotype III (β-tubulin III, mouse monoclonal; Sigma) for neurons, anti-GFAP (rat monclonal; Sigma) for astrocytes, Ricinus communis agglutinin-1 for microglia (biotinylated RCA-1; Sigma) and anti-galactocerebroside (GalC) for oligodendrocytes. Mouse monoclonal antibody specific for GalC was obtained from a hybridoma cell line grown in our laboratory (16). Immunocytochemistry was performed according to the methods described previously (17).

As it is difficult to obtain a sufficient number of human CNS neurons for experimental neurologic studies, A1 human hybrid neuronal cell line was used in the assay of neuronal response. A1 human neuronal cell line has been generated by somatic fusion between a human fetal cerebral neuron and a human SK-SH-SY5Y-A4 neuroblastoma cell with HGPRT deficiency (18).

Reverse transcription-polymerase chain reaction

Total RNA was extracted using TRIzol reagent from the cultures of astrocytes or A1 cells (GIBCO-BRL, Gaithersburg, MD, USA). Complimentary DNA (cDNA) templates from each sample were prepared from 2 μg of total RNA primed with oligo dT primers (Pharmacia, Gaithersburg, MD, USA) using 400 U of MMLV reverse transcriptase (GIBCO-BRL). Five microliters of each cDNA product was amplified by 30 PCR cycles (94°C for 30 s, annealing at 60°C for 60 s and extension at 72°C for 90 s). The primer sets were: 5′-TAATTGGTTCCATGGCCGGGCCC-CTGCGCG-3′ sense and 5′-ATTAAGGATCCC-

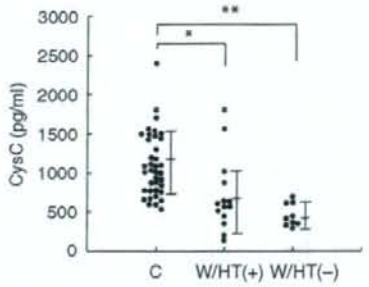

Figure 1. Cystatin C concentrations in the cerebrospinal fluid of patients with WML grade 0–1 (C), WML grade 3 with hypertension [W/HT(+)] and WML grade 3 without hypertension [W/HT(−)]. Cystatin C concentrations were measured using the method described in Materials and methods. The vertical bars indicate mean ± SD. The mean CSF cystatin C levels were decreased in patients with W/HT(+) and W/HT(−) compared with C. $*P = 0.0022$, $**P < 0.0001$.

CTAGGCGTCCTGACAGGTG-3′ anti-sense for cystatin C and 5′-CCATGTTCGTCATGGGTG-TGAACCA-3′ sense and 5′-GCCAGTAGAGGC-AGGGATGATGTTC-3′ anti-sense for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR was carried out in 50 μl of reaction mixture containing Taq DNA polymerase buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 200 mM dNTP, 2.5 mM $MgCl_2$, 0.5 mM of each primer) and 2.5 U of Taq DNA polymerase (GIBCO-BRL). To confirm the purity of cell preparations, cDNA from each cell type-enriched culture was amplified for neurofilament-L (NF-L, a cell type-specific marker for neurons), GFAP (for astrocytes), CD11b (for microglia) and myelin basic protein (for oligodendrocytes). Ten microliters of the reaction mixture was analyzed by 1.5% agarose gel in the presence of ethidium bromide.

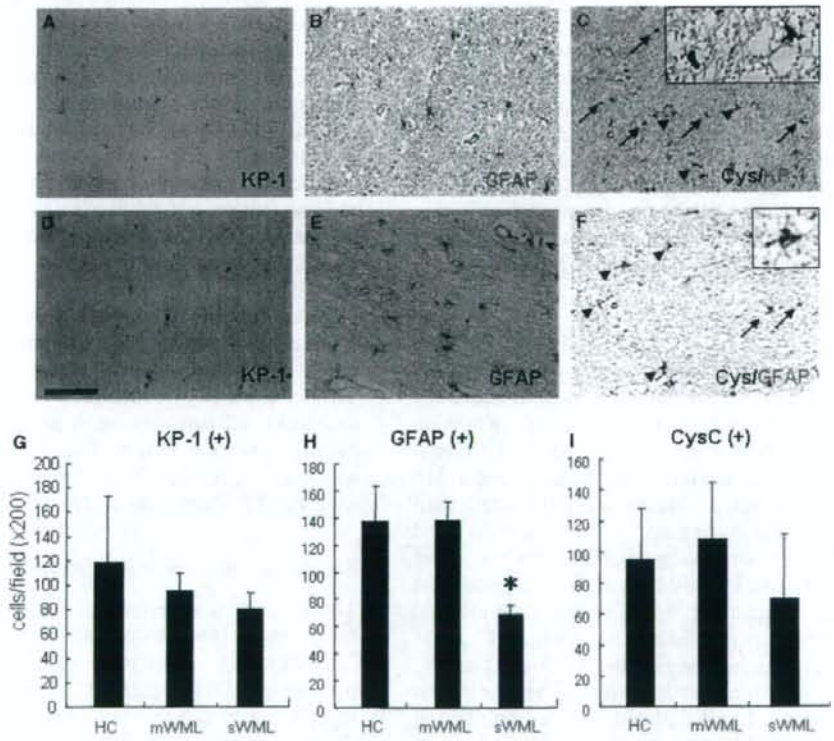

Figure 2. Immunoreactivity for KP-1, GFAP and cystatin C in the brain white matter of patients without CVD (HC), CVD patients with mild WML (mWML) or patients with severe WMLs (sWML) (×200 magnification). (A) KP-1 staining and (B) GFAP staining in sWML. (C) Double immunostaining for cystatin C with nickel ammonium sulfate (arrowheads), and for KP-1 with DAB (arrow) show that both immunoreactivities were localized in different cells. Round-shaped KP-1-positive cells (left) and star-shaped GFAP-positive cells (right) are shown in higher magnification (×400) in the upper-right corner. (D) KP-1 staining and (E) GFAP staining in mWML. (F) Double immunostaining for cystatin C with nickel ammonium sulfate (arrowheads), and for GFAP with DAB (arrow) indicate that some cells are both cystatin- and GFAP positive, but other cells are only GFAP positive. A double-positive cell is shown in higher magnification (×400) in the upper-right corner. (G) KP-1-positive, (H) GFAP-positive and (I) cystatin C-positive cells were counted in three different fields with ×200 magnification. $*P < 0.05$ compared with control or mWML.

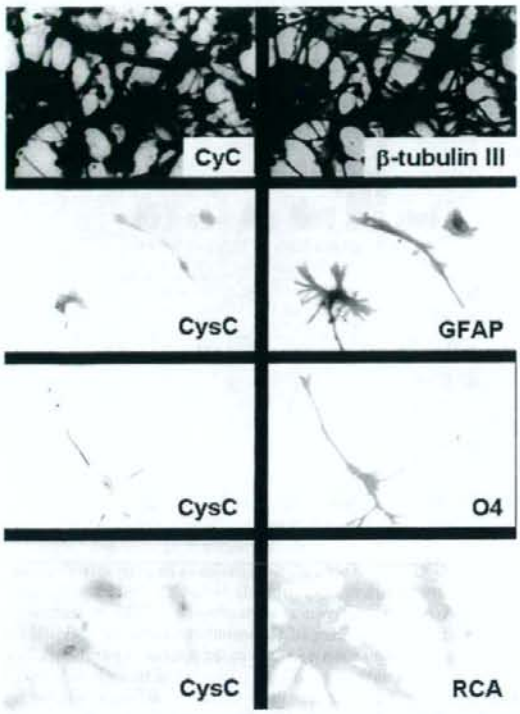

Figure 3. Immunocytochemical analysis for the detection of cystatin C (A, C, E and G) in each cell type-specific cultures of human neurons (B with β-tubulin III staining), astrocytes (D with GFAP staining), oligodendrocytes (F with GalC staining) and microglia (H with RCA-1 staining). Double staining was performed with incubation with primary antibodies followed by secondary antibodies conjugated with Texas-red or fluorescein-4-isothiocyanate (FITC). Cystatin C was detected in the cytoplasm of all kind of cells. Microglia had a patchy or granular staining and the other cell types had even staining in the cytoplasm.

Statistical analyses

Data are presented as mean values ± SEM. The statistical significance between group comparisons was determined by Student's *t*-test or a one-way ANOVA and Student–Newman–Keuls test using the StatView program (SAS, Cary, NC, USA). A value of $P < 0.05$ was considered statistically significant.

Results

CSF cystatin C levels

By means of the established ELISA method, CSF cystatin C levels were compared among the groups of control, W/HT(+) and W/HT(−) groups. As shown in Fig. 1, mean CSF cystatin C levels were decreased in W/HT(+) as well as in W/HT(−) compared with that in control ($P = 0.0022$ and $P < 0.0001$ respectively). No significant difference was detected between W/HT(+) and W/HT(−). The range of values in control was not different from the healthy control in our previous study (9, 10).

Cystatin C expression in WMLs

Rarefaction of white matter was seen in the group of WML(+) with conventional stainings. Small incomplete infarctions were sometimes recognized in the sWML group. In a patient with CAA, amyloid deposition was detected in the vessels of leptomeninges and cortical surface. Hyaline degeneration of the perforating arterioles was common in the other two patients with sWML. Because a regressive change of astrocytes and an activation of microglia are characteristic features of WMLs in Binswanger's disease (19), expression of cystatin C was immunohistochemically evaluated in those cells. The number of KP-1(+) microglial cells was not different among control, mWML and sWML (Fig. 2A, D and G). The number of astrocytes were significantly decreased in sWML compared with either in control ($P = 0.0027$) or in mWML ($P = 0.0024$) (Fig. 2H). Cystatin C immunoreactivity was localized in the cytoplasm of astrocytes and was not coincident with KP-1 immunoreactivity (Fig. 2C and F).

Localization of cystatin C in human-derived cultured cells

After each type of neural cells was isolated in cultures by the established method, cystatin C immunoreactivity was investigated with double immunostaining. Cystatin C was detected in neurons, astrocytes, oligodendrocytes and microglia with each cell type-specific marker (Fig. 3).

Secretion of cystatin C in cultures of human astrocytes and A1 human hybrid neurons

As immunoreactivity for cystatin C was mainly detected in astrocytes in WMLs, cystatin C levels in astrocytes and A1 neurons as a control were investigated by an ELISA (Fig. 4). Proteases, such as thrombin and MMP-2, induced cystatin C gene expression more prominently, when compared with pro-inflammatory cytokines in astrocytes. Thrombin was the strongest inducer for cystatin C in both astrocytes and neurons. Protein levels of cystatin C in culture supernatant or in cell lysate were then analyzed in cultures of astrocytes or A1 neurons. Thrombin markedly increased cystatin C levels in

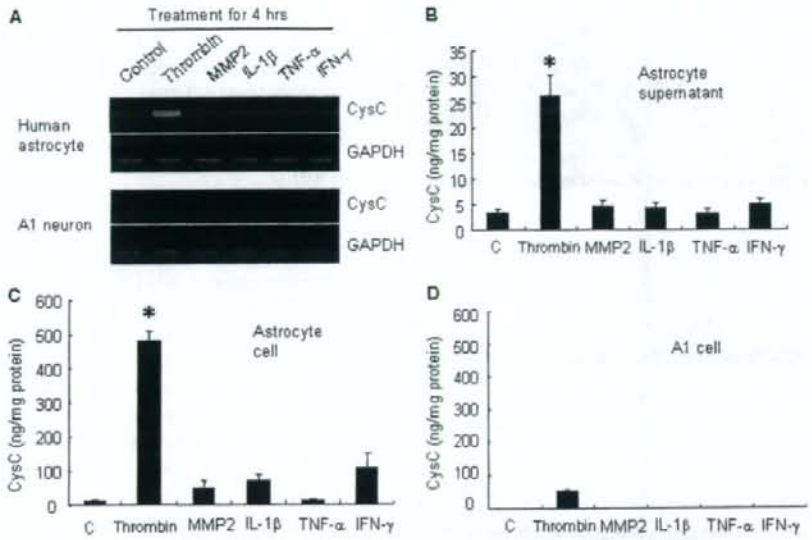

Figure 4. Effect of proteases and proinflammatory cytokines on cystatin C mRNA and protein expression in human astrocytes or A1 hybrid neurons. (A) Human astrocytes and A1 neurons were incubated in a medium containing 5 U/ml of thrombin, 0.5 μg/ml of MMP-2, 10 ng/ml of IL-1β, TNF-α or IFN-γ for 6 h. Then, total RNA of the cells was analyzed by RT-PCR as described in Materials and methods. GAPDH (251 bp) was used as an expression control. Treatments of thrombin, MMP-2 or IL-1β induced cystatin C gene (463 bp) in astrocytes and A1 neurons. The figure shows a representative of three independent experiments. (B–D) Cystatin C production by human astrocytes or A1 neurons after 48 h of treatment with or without 5 U/ml of thrombin, 0.5 μg/ml of MMP-2, 10 ng/ml of IL-1β, TNF-α or IFN-γ. The amounts of cystatin C in culture supernatants or in cell lysate was determined using an established ELISA method and corrected for the amount of the cell protein levels. (B) Astrocyte supernatant, (C) Astrocyte cell lysate, (D) A1 cell lysate. Values are mean ± SEM ($n = 3$). $^*P < 0.05$.

the cell lysates of both astrocytes and neurons. Cystatin C was not detected in the culture supernatant of A1 neurons; only astrocytes secreted cystatin C in the supernatant.

Discussion

In the healthy brain, cystatin C is stably localized in the choroid plexus, leptomeningeal cells, glial cells and neurons (20–22). However, in the diseased state of animal brains, cystatin C protein expression increases in the hippocampal neurons after the events of stroke/ischemia (23) and status epilepticus (24). Rat facial nerve axonotomy also increased cystatin C expression in the microglia surrounding the facial nerve nucleus (25).

On the contrary, this study revealed that cystatin C levels in the CSF were significantly decreased in the patients with sWML. Our previous studies have demonstrated that cystatin C levels were also low in patients with leptomeningeal metastasis (9) and neurologic inflammatory diseases, such as multiple sclerosis and Guillain–Barré syndrome (10). Cystatin C is an inhibitor of cysteine proteases including cathepsins-B, -H, -L and -S, and cystatin C degradation under the condition of high protease activities in pathological conditions was thought to be the reason for the low levels in the CSF. As assay of cathepsin B activity in all the patients in this study found no elevation in the enzyme activity, there might be other reasons for the low cystatin C levels, such as focal and chronic elevation in cathepsin activities due to change in microenvironment. It was reported that cystatin C levels were decreased in patients with CAA, in which amyloid β protein and cystatin C were co-deposited in the vessels of leptomeninges and cortical surface (7, 8). Although in this study, this type of CAA was suspected in the elderly patients with W/HT(−), there was no significant difference in cystatin C levels found between W/HT(+) and W/HT(−).

The immunohistochemical feature observed in this study was the decrease in the number of astrocytes in the severe WMLs, although rarefaction of white matter is common. The findings were compatible with the results reported in the pathology of cerebrovascular disease and AD (19). Positive immunoreactivity of cystatin C was found in all the cell types, such as neuron, astrocytes, oligodendrocytes and microglia in primary human neural cell cultures. However, in

WML brain sections, cystatin C expression was only detected in astrocytes. It has been hypothesized that cystatin C has multiple functions and contributes to the neurogenesis and neuronal degeneration. The glycosylated form of cystatin C is critical for the mitotic activity of fibroblast growth factor-2 in neural stem cells (26). A previous study with transient forebrain ischemia in rats indicated that cystatin C immunoreactivity was localized in morphologically degenerative neurons in the hippocampus suggesting that cystatin C is involved in cell survival *in vivo* (23). Cystatin C expression in astrocytes of sWML group appears stronger than that in the control groups, and the number of cystatin C-positive cells is not statistically different among the three groups despite that the number of astrocytes is decreased in the severe WML group. These results suggest that the expression of cystatin C in the regressive astrocytes is upregulated in the process of white matter degeneration.

It is worth noting that thrombin treatment dramatically increased the expression and secretion of cystatin C in astrocyte cultures dose dependently and that thrombin in high doses induced cell death. Thrombin is known to induce astrocyte stellation in morphology through the specific receptor, protease-activated receptor 1, and behave neuroprotectively by secreting glutathione peroxidase (27, 28). Upregulation of cystatin C is possibly one of the self-defense responses during neuroinflammation or neurodegeneration to inhibit protease release from lysosomes, where cystatin C localizes. As the involvement of MMP-2 and cytokines are known widely as the cause of ischemic WML formation (29–31), we treated human astrocytes cultures with MMP-2 and cytokines, such as IL-1β, TNF-α or IFN-γ to see if any of these molecules induces cystatin expression in astorocytes. However, none of these increased the production of cystatin C.

In the pathology of WMLs, blood–brain barrier (BBB) is disrupted as it progresses, which induces extravasation of serum proteins (32). Because of BBB dysfunction, thrombin might deposit in the brain (33) or endogenous prothrombin and thrombin would work toxic in the CNS (34, 35). Our immunohistochemical study did not reveal the difference in thrombin immunoreactivity among groups, but it is possible that a continuous exposure to thrombin might exist in the area around WML. The results of this study suggest that astrocytes are the main source of secreted cystatin C in the CNS and a decreased number of astrocytes in WML leads to low concentrations of cystatin C in the CSF.

In conclusion, it is the first time that the decreased CSF cystatin C levels in patients with sWML were reported when compared with patients with mWML and controls. Our study suggests that low levels of CSF cystatin C in ischemic WMLs are due to the decreased number of astrocytes that secrete cystatin C in response to the stimuli of proteases and inflammatory cytokines. As cystatin C secretion from astrocytes may influence cystatin C levels in CSF, further examination should be conducted to determine whether cystatin C modifies the pathological progression of WMLs.

Acknowledgements

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Original Article

Effect of Keishibukuryogan on Silent Brain Infarction over 3 Years

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無症候性脳梗塞に対する桂枝茯苓丸の3年間投与後の効果

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Original Article

Effect of Keishibukuryogan on Silent Brain Infarction over 3 Years

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Abstract

The purpose of this study was to evaluate the effect of keishibukuryogan (KB) against the cognitive symptoms associated with silent brain infarction in a prospective cohort study. The subjects were 93 patients with silent brain infarcts who visited the Department of Japanese Oriental Medicine, University of Toyama, and its allied hospitals. They consisted of 24 males and 69 females, mean age (± S.E.) 70.0±0.8.Group SK (n=51) consisted of patients who used KB extract for more than 6 months per year. Group SC (n=42) consisted of patients who did not use Kampo formulas. The NS group (n=44) consisted of elderly subjects who had no silent brain infarction, 21 males and 23 females, with a mean age (± S.E.) of 70.7±0.7 years. Among the three groups, the revised version of Hasegawa's dementia scale, apathy scale and self-rating depression scale were compared between the study start and after three years. In the SK and SC groups, these scores, and the subjective symp-

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tom levels (head heaviness, headache, dizziness or vertigo, stiff shoulder) were also studied. The results showed that the self-rating depression scales at study start for the SK and SC groups were significantly higher compared to the NS group. In spite of the scores for the NS group increasing after three years, the SK group scores were significantly decreased compared to the SC and NS groups. KB was effective against head heaviness, which often complicates silent brain infarction. In the above mentioned, KB was effective in treating cognitive disorders and subjective symptoms related to silent brain infarction.

Key words: keishibukuryogan, silent brain infarction, depression, revised version of Hasegawa's dementia scale, apathy scale, self-rating depression scale

要旨

無症候性脳梗塞患者に対する桂枝茯苓丸を主体とした漢方薬の効果を3年間にわたり前向き研究により検討した。対象は富山大学附属病院ならびに関連病院を受診した無症候性脳梗塞患者93名で男性24名，女性69名，平均年齢70.0±0.8才である。桂枝茯苓丸エキスを1年あたり6カ月以上内服した51名をSK群，漢方薬を内服せずに経過を観察した42名をSC群とし，MRI上明らかな無症候性脳梗塞を認めない高齢者44名，平均年齢70.7±0.7才をNS群とした。3群間において，開始時と3年経過後の改訂版長谷川式痴呆スケール，やる気スコア（apathy scale），自己評価式うつ状態スコア（self-rating depression scale）を比較した。また，SK群とSC群においては自覚症状（頭重感，頭痛，めまい，肩凝り）の経過も比較検討した。その結果，3群間の比較では，自己評価式うつ状態スコアにおいて開始時のSK群とSC群は，NS群に比べて有意にスコアが高かった。しかし，3年経過後にはNS群は開始時に比較し有意に上昇したが，SK群は有意に減少した。さらに無症候性脳梗塞にしばしば合併する自覚症状の頭重感において桂枝茯苓丸は有効であった。以上の結果から，無症候性脳梗塞患者の精神症状と自覚症状に対して桂枝茯苓丸が有効である可能性が示唆された。

キーワード：桂枝茯苓丸，無症候性脳梗塞，うつ症状，改訂版長谷川式痴呆スケール，やる気スコア，自己評価式うつ状態スコア

Ⅰ. Introduction

Silent brain infarction is diagnosed by magnetic resonance imaging (MRI) and computed tomography (CT) as small cerebral infarctions without neurological symptoms. Silent brain infarction is thought to have a vascular origin and is frequently seen in neurologically asymptomatic elderly patients. It was recently reported that cerebral stroke and vascular dementia are related to silent brain infarction[1]. Silent brain infarction is characterized by the mental symptoms of lowering of the function of acknowledgment[2] and a state of depression[3]. In terms of prevention by Western medicine, anticoagulant therapy was not able to suppress its advance[4], and the only treatment available is the control of blood pressure to prevent cerebral infarction by hypertension[5].

Keishibukuryogan (KB) is a Kampo (Japanese herbal) formula that improves the microcirculation[6]. Clinically, KB has been reported to have strong hemorheological and anti-coagulative effects[7]. KB was also demonstrated to have an antioxidant effect[8] and a hypotensive effect in spontaneously hypertensive rat[9]. Taken together, it is suggested that KB has a salutary effect on silent brain infarction. In an earlier study, we demonstrated that KB affected mental symptoms of silent brain infarction in the short term[10]. Therefore, in this study we present the results of a prospective cohort study that examined the long-term effects of KB on mental symptoms compared to patients with silent brain infarction but not treated with Kampo formulas and to healthy elderly subjects without silent brain infarction.

Ⅱ. Subjects

Patient selection :

1) Neurologically normal patients were diagnosed with silent brain infarction based on high-intensity lesions greater than 3 mm in size on T_2-weighted images that coincided with low-intensity lesions on T_1-weighted images on MRI.
2) Patients who had severe dementia, complications from other severe diseases, or who were judged inappropriate for this study by the investigators were excluded from entry. Informed consent was obtained from all patients prior to enrollment according to our institutional guidelines.

Ⅲ. Methods

1) Study protocol :

We selected the SK group according to our previous study. It was composed of patients for whom administration of KB had previously shown favorable results[10]. SK subjects used KB for more than 6 months per year and were free of side effects. The

Table 1 Patient characteristics

Group		SK	SC	NS
Sex	Male	11	13	21
	Female	40	29	23
Age	(Years, mean ± S.E.)	70.0 ± 0.9	69.9 ± 1.4	70.7± 0.7
Complication	None	16	15	14
	Hypertension	26	22	27
	Diabetes mellitus	6	6	6
	Hyperlipidemia	16	14	10
Number of high intensity areas of brain by MRI	Single	19	20	–
	Multiple	32	22	–

Table 2 Revised version of Hasegawa's dementia scale (HDS-R), apathy scale and self-rating depression scale (SDS)

		Beginning point	3 years
HDS-R	SK	27.3±0.3	27.4±0.3
	SC	27.5±0.4	27.8±0.3
	NS	26.8±0.5	27.5±0.4
Apathy scale	SK	11.4±0.9	10.7±0.9
	SC	12.8±1.4	12.6±1.5
	NS	13.8±0.7	13.8±0.8
SDS	SK	39.2±1.4[c]	36.2±1.4[b]
	SC	41.5±1.7[c]	41.9±1.9
	NS	32.2±1.1	35.5±1.2[a]

[a] $p<0.01$ and [b] $p<0.05$ vs. corresponding beginning point; [c] $p<0.01$ vs. NS group in beginning point.

SC group was composed of patients who had silent brain infarction but did not receive Kampo treatments. They visited the Department of Japanese Oriental Medicine, University of Toyama, and its allied hospitals. The NS group consisted of elderly subjects who had no silent brain infarction and had been observed for 3 years in a health-screening program of the brain at the Shimane Institute of Health Science. Patients in the SK group were administered KB extract (made by Tsumura & Co. 7.5 g/day : n= 33, made by Kanebo LTD. 6.0 g/day : n=18) between meals three times a day. The revised version of Hasegawa's dementia scale (HDS-R)[11], apathy scale[12], and self-rating depression scale (SDS)[13] were assessed by the investigators at the beginning and after 3 years of medication administration. In the SK and SC groups, subjective symptoms (heaviness of head, headache, dizziness or vertigo, stiff shoulder) were evaluated by the investigators at the beginning and after 3 years of medication administration by means of a 5-point rating scale (0=no symptoms, 1=very slightly affected, 2=slightly affected, 3=moderately affected, 4=severely affected). Further, in the SK group, Terasawa's Oketsu score[14] was evaluated at the beginning and at 3 years.

2) Trial period : June 1999 to May 2006.

3) Statistical analysis : Data are shown as mean ± S.E. Two-way repeated-measures ANOVA, Mann-Whitney U test and Student's t-test were used for statistical analysis, and $p<0.05$ was considered significant.

Ⅳ. Results

Patient characteristics (Table 1)

The total enrollment consisted of 127 subjects with silent brain infarctions. 74 patients were treated

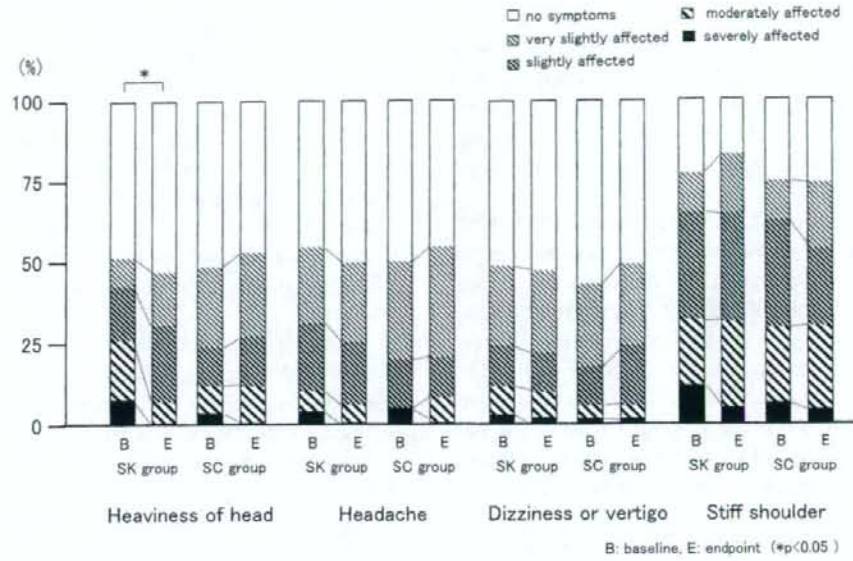

Figure 1 The rate of subjective symptoms at baseline and endpoint in patients with silent brain infarction

with keishibukuryogan (SK group) and 53 patients were treated without the use of Kampo formulas (SC group). In the 3-year study period, none of the patients in either group had a stroke. Discounting the patients who dropped out or whose various scores could not be assessed at 3 years, 51 patients in the SK group and 42 patients in the SC group were finally analyzed. The SK patients took KB extract for 11.1±0.2 months/year on average. There were no statistical differences between the SK and SC groups in terms of gender, age, complications and degree of infarction. The NS group consisted of 44 subjects, 21 males and 23 females (Table 1).

Revised version of Hasegawa's dementia scale (Table 2)

Mean HDS-R was 27.3±0.3 at the beginning and 27.4±0.3 at 3 years in the SK group, and 27.5±0.4 at the beginning and 27.8±0.3 at 3 years in the SC group. Mean HDS-R was 26.8±0.5 at the beginning and 27.5±0.4 at 3 years in the NS group. There was no statistical significance among the three groups.

Apathy scale (Table 2)

The mean apathy scale in the SK group was 11.4 ±0.9 at the beginning and 10.7±0.9 at 3 years, and in the SC group 12.8±1.4 at the beginning and 12.6 ±1.5 at 3 years. In the NS group, the mean apathy scale was 13.8±0.7 at the beginning and 13.8±0.8 at 3 years. There was no statistical significance among the three groups.

Self-rating depression scale (Table 2)

The mean SDS scale in the SK group was 39.2± 1.4 at the beginning and 36.2±1.4 at 3 years, and in the SC group 41.5±1.7 at the beginning and 41.9± 1.9 at 3 years. In the NS group, mean SDS was 32.2 ±1.1 at the beginning and 35.5±1.2 at 3 years. At the beginning, mean SDS values of the SK and SC groups were significantly higher than that of the NS group ($p<0.01$). After 3 years, mean SDS of the SK group was significantly lower than at the beginning ($p<0.05$),and among the three groups, mean SDS of the SK group was also significantly decreased compared with that of the SC and NS groups ($p<0.05$).

Subjective symptoms (Figure 1)

The patients with silent brain infarction often have combined symptoms of heaviness of head, headache and dizziness. Progression of the symptoms was compared between the SK and SC groups. In the SK group, heaviness of head was improved after 3 years compared to the beginning point. Headache, dizziness or vertigo and stiff shoulder did not change over 3 years in either group, and there were no statistical significance in the two groups. Terasawa's Oketsu score was 34.0±3.4 at the beginning and 36.7±3.7 at 3 years in the SK group, not a significant change.

V. Discussion and conclusion

In an earlier study, we demonstrated that KB had short-term effects on mental symptoms in silent

brain infarction[10]. Herein, we examined the long-term effects of KB on mental symptoms over 3 years, and compared to the subjects without Kampo treatment. After 3 years, we also compared the results against those of elderly subjects who were observed by a health-screening program of the brain for 3 years.

On the function of acknowledgement, HDS-R did not change among the three groups between the beginning and after 3 years. There were some reports about the possible relationship of silent brain infarction with the cognitive function, with the disagreement in their results being ascribed to age differences of the subjects, extent of infarction, and examination method of the cognitive function. It was reported that silent brain infarction and the cognitive function are more frequent in elderly people[15] and are also related to a higher degree of ventricular hyperintensity[16]. We examined these values with clinical assessments in the current study.

For emotional disorders, we studied the apathy scale, an index of the lowering of desire, and SDS, an index of the depressive state. For the apathy scale, there was no difference among the three groups at the beginning and after 3 years. This scale reflects neuronal degeneration, shows that reduction after a stroke[17] is independent of the depressive state[18], and is related to decreased blood flow in the frontal lobe[19]. Therefore, patients with a slight lesion like silent brain infarction were not differentially recognized compared with elderly subjects.

On SDS, it is reported that silent brain infarction is related to a tendency toward depression[3]. In this study, the mean SDS values of the SK and SC groups were significantly higher than that of NS group at the beginning of the study. After 3 years, mean SDS of the elderly had worsened significantly, but that of the SK group had improved significantly compared with the value at the beginning. Thus, comparing SK patients with the other groups, SDS of the former improved significantly after 3 years. As for the anti-depression effect of KB, firstly, it has been suggested to work by causing improvement in blood circulation[6,7], as decreases in cerebral blood circulation causes silent brain infarction[20]. Secondly, there are some reports that KB exerts favorable effects on the mental state directly[21], so the depressive state is thought to improve.

Concerning subjective symptoms, silent brain infarction is reportedly related to non-specific symptoms such as headache, etc.[22]. In the present study, 50-70% of the patients had non-specific symptoms. KB was useful for improving some symptoms complicating silent brain infarction. Especially in regard to heaviness of head, KB showed improvement compared with the subjects treated without Kampo formulas.

The Oketsu score did not show a significant change after 3 years. Because this score worsens by aging, it is thought to need a comparison with control subjects. Further, as this score was scattered among the institutions in this study, this point is thought to be a problem awaiting solution in the future.

Finally, KB may be useful against depression and subjective symptoms, especially heaviness of head, in relation to silent brain infarction. It is clear that future investigations will need to study the functional mechanisms of KB and to focus on much larger study populations as well as on longer-term longitudinal studies to confirm the protective effects of KB against cerebral attack and vascular dementia.

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