The Effects of Choto-san on the mRNA Expression of Alzheimer’s Disease Related Factors in the Permanent Ischemic Rat Brain

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Choto-san is a Kampo medicine that has been used clinically for the treatment of dementia. We measured the mRNA expressions of some factors related to Alzheimer’s disease in a dementia model rat brain. The expressions of β-amyloid precursor protein, γ-secretase, α7 nicotinic acetylcholine receptor, neprilysin, and insulin degrading enzyme (IDE) were significantly increased on day 4 after permanent occlusion of the bilateral common carotid arteries (2VO). Choto-san inhibited the enhancement of IDE expression caused by 2VO, although it failed to show any effects on the expressions of the other molecules. These results suggest that Choto-san may produce a state in which it is not necessary to induce IDE expression to demonstrate the anti-dementia effects.

Key words Choto-san; Alzheimer’s disease; neprilysin; mRNA expression

Permanent occlusion of the bilateral common carotid arteries (2VO) in rats is a chronic cerebral hypoperfusion model, and this model causes progressive and long-lasting cognitive deficits,¹ and progressive neuronal damage in the hippocampus and the white matter.² The impairment of cognitive function and the pathophysiology observed in this model are similar to the pathogenesis in Alzheimer’s disease (AD) and cerebrovascular disease,³,⁴ suggesting that this 2VO model is useful for investigating molecular mechanisms of the generation of dementia.

Various factors that play an important role in the mechanism of AD have been isolated. We have reported previously that the gene expressions of amyloid precursor protein (APP), γ-secretase, and α7 nicotinic acetylcholine receptor (α7NicR) are significantly increased on day 4 after the 2VO operation.⁵ Choto-san is one of several Kampo medicines (Wakan-yaku) that have been used clinically for the treatment of dementia. The anti-dementia effects have been shown to be due to its anti-hypertensive, free radical scavenging, and anti-excitotoxic effects,⁶ however, its direct effects against AD factors have never been shown. We evaluated the changes in expression of APP and other related factors by Choto-san in the 2VO rat brain using a semiquantitative PCR protocol.

MATERIALS AND METHODS

Animals Male Wistar rats (Sankyo Labo Service, Hamamatsu, Japan) aged 14 weeks were used. The animals were housed in rooms with a 12-h light/dark cycle (lights on from 7:30 a.m. to 7:30 p.m.) at a room temperature of 24±1°C with a relative humidity of 55±5%. Food and water were supplied ad libitum.

Extract Preparation Choto-san water extract was prepared from a mixture of 11 medicinal plants according to the following recipe: Aurantii Nobilis pericarpium (Peel of Citrus unshiu Markovitch) 3 g, Ophiopogon japonicus (root of Ophiopogon japonicus Ker-Gawler) 3 g, Pinelliae tuber (tuber of Pinellia ternata Breitenbach) 3 g, Hoelen (fungus of Poria cocos Wolf) 3 g, Uncariae Ramulus et Uncus (hooks and branch of Uncaria rhynchophylla Miquel, Uncaria sinensis Oliver) 3 g, Ginseng radix (root of Panax ginseng C.A. Meyer) 2 g, Saposhnikoviae radix (root and rhizome of Saposhnikovia divaricata Schischkin) 2 g, Chrysanthemum morifolium Ramat-Ulle, Chrysanthemum indicum Linne) 2 g, Glycyrrhiza radix (root of Glycyrrhiza uralensis Fisher, Glycyrrhiza globa Linne) 1 g, Zingiberis rhizoma (rhizome of Zingiber officinale Roscoe) 1 g, and Gypsum Fibrosum (CaSO₄·2H₂O) 5 g. All of the components except Ramulus et Uncus were combined and boiled in 280 ml of water for 60 min. Romulus et Uncus was added after 45 min and the boiling was continued for a further 15 min. The water extract was filtered and then freeze-dried.

2VO Operation The surgery was performed as described previously.¹³ Rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.). A ventral midline incision was conducted to expose the common carotid arteries. The arteries were then carefully separated from the adjacent vessels and nerves. Silk suture (No.1, Natsume Co. Ltd., Tokyo, Japan) was used to occlude the arteries. Sham-operated controls under went an identical operation without occlusion of the arteries.

Drug Administration No drugs were administered during the first 24 h after the 2VO operation. The sham-operated rats and the 2VO-Water group rats were given water, while the 2VO-Choto-san group rats were orally administered 1 g/kg Choto-san once a day around 4:00 p.m. for three consecutive days. The animals were decapitated 18 h after the final drug administration on day 4. The whole brains were removed and frozen in liquid nitrogen and then kept at −80°C for further RNA isolation.

RNA Isolation and RT/PCR Total RNA isolation was performed using Isogen® (Nippon Gene, Toyama, Japan), which is a modification of the methods described by Chomczynski and Sacchi.⁷ The RNA was precipitated with isopropanol. The precipitate was resuspended in DEPC-water. The RNA concentration was spectrophotometrically measured at 260 nm. First strand cDNA was synthesized using 200 units of Superscript II reverse transcriptase (Invitrogen) from 2.0 μg of total RNA and 0.5 μM oligo(dT) primer in a 20 μl mixture. Polymerase chain reaction was carried out in
10 μl reaction mixtures containing 1 μl of the first strand cDNA, 1 μl of sense and antisense primers, 250 μM dNTPs, and 2 units of Taq polymerase (Promega, Madison, WI, U.S.A.) containing 2.5 mM MgCl₂. Thermocycling was performed using the following protocol: first, 72 °C for 10 min, second, designated cycles of 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min, and third, 72 °C for 10 min. The primers used in the polymerase chain reaction are shown in Table 1. The suitable PCR cycles, also shown in Table 1, were determined by carrying out the PCR at many cycles as described previously.3

**Data Analysis** All results are expressed as the mean±S.E.M. Statistical significance between different groups was analyzed by one-way analysis of variance (ANOVA). Differences of p<0.05 were considered significant.

### RESULTS

In the 2VO-Water group, which underwent the 2VO operation and received water, increased expression of APP, γ-secretase and α7NicR was observed as reported before.5 The expression of neprilysin was dramatically increased on day 4 after the 2VO operation to a level double that of the sham group. Up-regulation of IDE expression following the 2VO operation to a level double that of the sham group was analyzed by one-way analysis of variance (ANOVA). Differences of p<0.05 were considered significant.

The effects of Choto-san on the 2VO-induced expression changes in AD related factors were examined at 1.0 g/kg, a dose at which improved effects on water maze task in mice,4 hypertension in SHR rats5 and prolongation of the thipental-induced sleeping time in rats10 have been observed. Choto-san showed an inhibitory effect on IDE enhancement caused by the 2VO operation, while APP, α7NicR, and neprilysin did not show any changes compared to the 2VO-Water group.

### DISCUSSION

We previously reported that the mRNAs expressions of the AD related factors APP, γ-secretase, and α7NicR were stimulated on day 4 after the 2VO operation.5 This stimulated our interest in the effects of Choto-san on these expression changes. Unfortunately, Choto-san did not change the mRNA expressions of these factors.

In this study, we found that the expression of neprilysin, a major degrading enzyme of amyloid β protein (Aβ), was dramatically increased, even though neprilysin expression is decreased in patients with sporadic AD.11 In a previous report, we discussed the functional role of up-regulation of mRNA of the APP/α7NicR system at an early stage of permanent ischemia. Recently, it has been reported that Aβ42, but not Aβ40, stimulates neurogenesis in neuronal stem cells from hippocampus in B16 mice.12 Based on this, we hypothesized that the transient enhancement expression of the APP/α7NicR system may play a role in protecting or compensating for the neuronal damage induced by ischemia.5 The increased Aβ42 may need to be degraded in order to protect the subsequent unwelcome event, that of formation of neuritic plaques. This may be the reason for the enhancement of neprilysin mRNA expression. Choto-san, however, did not change the level of neprilysin expression enhanced by 2VO-treatment.

The results of DNA array in our previous study showed that Choto-san induced gene expression changes in the 2VO rat brain.13 One factor which shows an expression change is...
IDE. IDE is also well known as an Aβ degrading enzyme.14,15) The expression was enhanced by 2VO and Choto-san inhibited this enhancement to the basal level. Since, as discussed above, the up-regulation of Aβ degrading enzyme may be a reflection of the protective or compensatory roles for neuronal damage, Choto-san may produce a state in which the induction of IDE expression is not necessary. Choto-san inhibited the expression enhancement of IDE, but not neprilysin, even though both are Aβ enzymes. Clarification of the differences in molecular functions and regulations between IDE and neprilysin may reveal the anti-dementia mechanisms of Choto-san. It has been reported that Uncariae Ramulus seems to be an active galenical of Choto-san on anti-dementia.8) We also reported that indol alkaloids of Uncariae Ramulus positively modulate serotonergic and cholinergic responses16) but inhibit the NMDA response,17) suggesting that alkaloids are involved in the effects of Choto-san/Uncariae Ramulus. However, it seems to get novel and interesting findings to identify the intrinsic functional factors if Choto-san and other “Wakan-yaku” use as a drug without divide galenical or compound like this report.

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