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Loss of cholinergic innervations in rat hippocampus by intracerebral injection of C-terminal fragment of amyloid precursor protein

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Abstract : The neurotoxicity of C-terminal fragments of amyloid precusor protein (CT) is known to play some roles in Alzheimer's disease progression. In this study, we investigated the effects of the recombinant C-terminal 105 amino acid fragment of amyloid precusor protein (CT105) on cholinergic function using CT105-injected rat. To study the effects of CT105 on septohippocampal pathway, choline acetyltransferase (ChAT) positive neurons were examined in the medial septum and in the diagonal band after an injection of CT105 peptide into the lateral ventricle. Immunohistological analysis revealed that the number of ChAT-immunopositive cells decreased significantly in both medial septum and diagonal band. In addition, CT105 decreased ChAT-immunopositive cells in the hippocampal area, particulary in the dentate gyrus. To study the effect of amyloid beta peptide (Aβ) and CT105 on the cholinergic system, each peptide was injected into the left lateral ventricle, and acetylcholine (ACh) levels were monitored in hippocampus. ACh level in the hippocampal area was reduced to 60% of control level in Aβ-treated group, and the level was reduced to 15% of control level in CT105-treated group, at one week after the injection. ACh level was further reduced to 35% of control in Aβ-treated group, whereas the level was slightly increased to 30% of control in CT105-treated group at 4 weeks after the injection. Taken together, the results in the present study suggest that CT105 impairs the septohippocampal pathway by reducing acetylcholine synthesis and release, which results in damage of learning and memory.

Keywords : Alzheimer's disease, beta-amyloid peptide, c-terminal 105 amino acid fragment of amyloid precusor protein, choline acetyltransferase

Introduction

The extracellular and intracellular deposits of amyloid beta peptide (Aβ) and hyperphosphorylated tau are the classical hallmarks of Alzheimer's disease (AD) observed at postmortem [18, 20, 33]. The progressive deterioration of learning and memory, which is one of the most devastating symptoms of the disease, is closely related to the loss of cholinergic function in the basal forebrain [1, 10, 11, 17]. Neurochemical and histological studies observed the reduced choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) enzyme activities in the cerebral cortex and hippocampus of AD brain [3, 21, 46]. Interestingly, the decrease of ChAT activity is occurred at the very early histopathologic stage of AD, before the onset of clinical symptoms [3].

Cholinergic afferents from the basal forebrain terminate diffusely within the molecular layers of the pyramidal and granular cells of the hippocampus. The cholinergic neurons of the septum, the nucleus basalis, and the diagonal band of Broca provide 90% of the cholinergic hippocampal innervations by axons projecting through the fimbria/fornix fiber bundle [16, 32, 39]. It has been reported that severe cholinergic deficits in AD brain may be induced by Aβ-induced toxicity followed by cerebral amyloidosis in the neocortex [5, 23]. Increasing evidence has been suggested that other cleaved products of amyloid precursor protein such as amyloidogenic C-terminal fragments (CT) might also be an important factor inducing neuronal death associated with AD [49]. Therefore, CT might also play some

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roles in one of the pathological events in the subcortical nuclei of the basal forebrain that project into the cortex and hippocampus. Previous study observed that a recombinant C-terminal fragment of amyloid precursor protein (CT105) induced learning and memory impairments by reducing acetylcholine synthesis [7]. With these previous findings, the effects of CT105 on the cholinergic alterations were investigated in the intracerebrally CT-injected rat models further through biochemical and pathological analyses in the present study.

Materials and Methods

Animal

Male Sprague-Dawley rats weighing 200-250 g were housed in a constant temperature room with a 12 h light-dark cycle. They were given feed and water *ad libitum*. All experiments were performed in accordance with the Guidelines for Animal Experiments of Ethics Committee of Cheju National University.

Chemicals

C-terminal 105 amino acid fragment of amyloid precusor protein (CT105) was generated as described previously [8]. $A\beta_{1-42}$ was purchased from U.S. Peptide (USA). Ethylhomocholine was obtained from Eicom (Japan). All other chemicals and drugs were purchased from Sigma-Aldrich (USA). All the reagents were prepared just before use.

Surgical procedure and CT105 treatment

The rats were anesthetized with equithesin (2 ml/kg, i.p.) and injected unilaterally CT105 (64 nmole) or $A\beta_{1-42}$ (64 nmole) into lateral ventrical using stereotaxic apparatus. Injections were made using a Hamilton syringe (26-gauge) at a rate of 1 μl/min. A needle was positioned at –0.8 mm posterior to Bregma and 1.4 mm lateral to the midline, and 3.6 mm ventral to the surface of the skull. The syringe was left in place for 5 min before slowly retracting it. Sham-operated animals were used for controls.

Quantification of the released acetylcholine level in the hippocampus

The probe was perfused with high K^+ Ringer's solution containing 50 mM NaCl, 100 mM KCl, 2.3 mM $CaCl_2$. AChE inhibitor, eserine sulfonate (200 μM) was added to the solution in order to obtain detectable quantities of acetylcholine (ACh) in the dialysate.

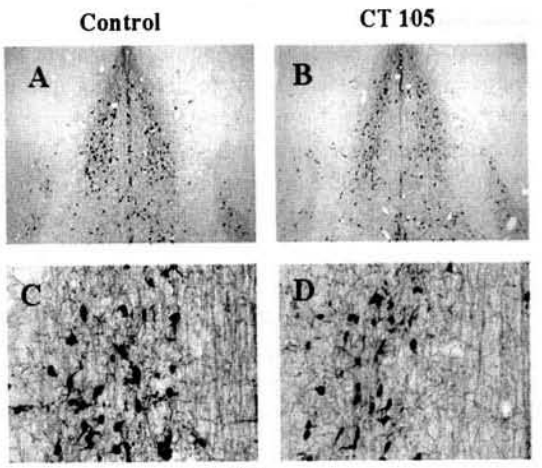
Dialysate was collected from hippocampus of rats in each group at one week and at four weeks after the injections. Samples were collected in tubes containing 1 μM ethylhomocholine, as an internal standard. Samples were injected directly into a column in which ACh was separated. An enzyme-reaction column is that contained immobilized AChE and choline oxidase, which converted ACh to hydrogen peroxidase. Hydrogen peroxidase was detected via a platinum electrode set at 450 mV. The mobile phase consisted of a 100 mM phosphate buffered solution contain 200 mg/l sodium 1-decanesulfonate and 65 mg/l tetramethylammonium chloride. The amount of ACh was quantified by HPLC system (SCL-10A; Shimadzu, Japan) with electrochemical detector (ECD-100; Eicom, Japan).

Tissue preparation.

Rats were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) and were perfused transcardially with 200 ml of phosphate buffered saline (PBS) containing heparin (5×10^4 units/ml), followed by 300 ml of 4% paraformaldehyde (PFA). The brains were removed, post-fixed for 24 h in 4% PFA, and thereafter transferred into 30% sucrose until they sank. Adjacent 30 μm sections including hippocampal area or medial septum and diagonal band were cut on a sliding microtome and stored at –20°C in a cryoprotecting buffer containing 25% ethylene glycol and 25% glycerin until use.

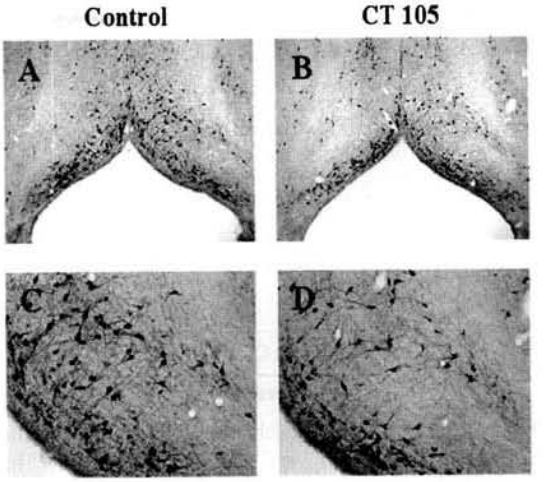
Choline acetyltransferase Immunohistochemistry

For ChAT labeling, free-floating sections were treated with 1% H_2O_2 in 10% ethanol for 30 min to block endogenous peroxidase. After washing, the sections were treated with 2 N HCl for 1 h at 37°C to denature DNA and then rinsed in borate buffer for 15 min (0.1 M, pH 8.4). They were preincubated in PBS containing 0.3% Triton X-100 and 2% of bovine serum albumin (blocking solution) for 30 min, and then incubated with shaking overnight at 4°C in mouse monoclonal anti-ChAT antibody (1 : 500 in PBS; Roche, Switzerland). Next, the sections were incubated for 1 h with biotinylated secondary anti-mouse IgG antibodies for 1 h at room temperature. The sections were incubated for 1 h in avidin-biotin-peroxidase complex



Medial septum

Fig. 1. The effect of CT105 on choline acetyltransferase expression in the medial septum. Immunoreactivity of ChAT in the medial septum of vehicle-treated (A, C) and CT105-treated rat (B, D). A, B: ×100, C, D: ×200.



Diagonal band

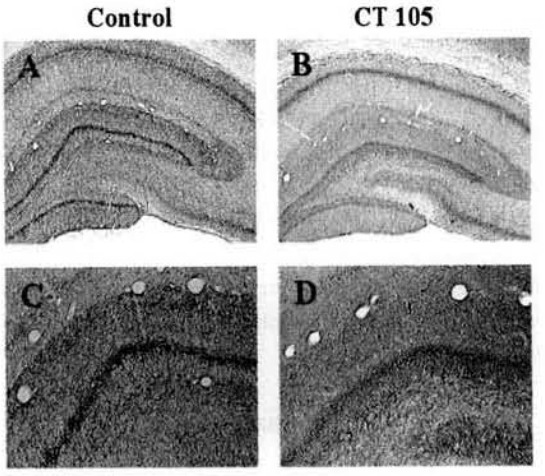
Fig. 2. The effect of CT105 on choline acetyltransferase expression in the diagonal band. Immunoreactivity of ChAT in the diagonal band of vehicle-treated (A, C) and CT105-treated rat (B, D). A, B: ×100, C, D: ×200.

(Vectastain Elite ABC kit; Vector Laboratories, USA), followed by peroxidase detection using 3,3-diaminobenzidine-tetrahydrochloride (DAB; Sigma, USA) as a chromogen (0.05% diaminobenzidine, 0.01% H_2O_2 in PBS). Sections were dehydrated through alcohols, cleared in xylene, and coverslipped in permanent mounting media. Peroxidase stained sections were examined with a light microscope (Olympus BX-60; Olympus, USA).

Results

Immunohistochemical analysis of ChAT positive neurons in the medial septum, the diagonal band, and the hippocampus

To study the effects of CT105 on septohippocampal pathway, ChAT positive neurons were examined in the medial septum and in the diagonal band after an injection of CT105 peptide into the lateral ventricle. The number of ChAT-immunopositive cells decreased significantly in the medial septum of the CT105-treated group compared to vehicle-treated group (Fig. 1). In addition, decreased number of ChAT-immunopositive cells was observed in the diagonal band of CT105-treated group (Fig. 2). Moreover, the abundance of ChAT-immunopositive cells decreased in the lateral zone of medial septum (Fig. 1) and in the vertical limb of the diagonal band (Fig. 2) of CT105-treated group. CT105 decreased ChAT-immunopositive cells in the hippocampal area, particulary in the dentate gyrus (Fig. 3).



Hippocampus

Fig. 3. The effect of CT105 on choline acetyltransferase expression in the hippocampus. Immunoreactivity of ChAT in the hippocampus of vehicle-treated (A, C) and CT105-treated rat (B, D). A, B: ×100, C, D: ×200.

Acetylcholine level in the hippocampus of Aβ or CT105-treated rat

To study the effect of Aβ or CT105 on the cholinergic system, each peptide was injected into the left lateral ventricle, and ACh levels were monitored

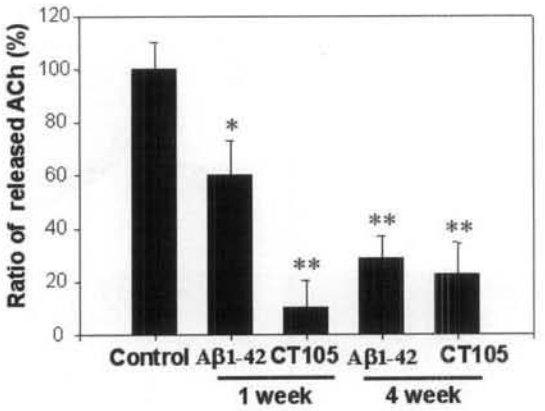


Fig. 4. Changes of acetylcholine (ACh) level in the hippocampus of $A\beta_{1-42}$ and CT105-injected rats. The released amounts of ACh were measured in $A\beta_{1-42}$ and CT-injected group at 1 and 4 weeks after an intracerebral injection. Dialysates were collected from hippocampus using microdialysis system and were quantitated with HPLC as described in Materials and Methods. The ACh level of each group (n = 4) was compared with the level of control group (100%). $*p < 0.05$ or $**p < 0.01$ vs. vehicle-treated group.

in hippocampus (Fig. 4). At one week after an injection, ACh level in the hippocampal area was reduced to 60% of control group ($p < 0.05$) in Aβ-treated group, and the level was reduced to 15% of control group ($p < 0.01$) in CT105-treated group, respectively. At four weeks after an injection, ACh level was further reduced to 35% of control ($p < 0.01$) in Aβ-treated group, whereas the level was slightly increased to 30% of control ($p < 0.01$) in CT105-treated group (Fig. 4).

Discussion

One of the consistent neurochemical features of AD is the loss of cholinergic neurons in the medial septum, the diagonal band of Broca, and the nucleus basalis magnocellularis [27]. A severe loss of ChAT in the neocortex and hippocampus and the degeneration of the cholinergic neurons are characterized in AD brain [4, 43, 44, 46]. Cholinergic deficits such as loss of ChAT activity in the cortex of AD brain are closely related to both cognitive and noncognitive behavioral changes in dementia [2, 40]. Moreover, the reduction of ChAT activity in the diagonal band of Broca and medial septal nucleus indicates that the degeneration or dysfunction of cholinergic neurons in the medial septal area and possibly neocortex is an important characteristic of AD [21]. Recently, to elucidate the neurodegenerative mechanism in cholinergic nervous system, injection of β-amyloid into lateral ventricle of rat has been used as an animal model for AD [23]. Infusion of Aβ into the cerebral ventricle in rats results in learning and memory deficits, cholinergic dysfunction, neuronal loss and deficiency of long-term potential [41, 51, 52].

With extensive evidence that Aβ may not be the sole active fragment contributing to AD pathogenesis, other potentially amyloidogenic products of APP such as CT have also been focused in relation to the pathological mechanism of AD [37, 55]. A number of *in vitro* and *in vivo* data have shown that CT can induce neurotoxicity through not only Aβ generation but also itself [31, 35, 38, 48, 54]. It has been reported that CT induces strong membrane inward currents, disruption of intracellular Ca^{2+} homeostasis, inflammatory reaction and render neuronal cells more vulnerable to Glu-induced excitotoxicity [15, 28-30]. Recent studies observed that an injection of CT into the brain of mice induces learning and memory impairments, neurochemical and neuropathological changes [7, 47].

Based on previous studies, to clarify the neurodegenerative changes in the cholinergic system in CT105-induced cognitive dysfunction, we observed the level of ACh release in the hippocampus of CT105-treated rat. Intracerebroventricular injection of CT105 significantly decreased ACh release in the hippocampus. Moreover, ChAT positive neurons are reduced markedly in the medial septum, diagonal band, and hippocampus. CT105 significantly reduced the release of ACh in the hippocampus. The data implicates that CT105 can modulate the release of endogeneous ACh in the cholinergic neurons and probably contribute to cognitive impairment. Previous study observed that Aβ protein inhibits ACh release and choline-uptake but does not affect ChAT activity in the hippocampus, supporting the vulnerability and sensitivity of basal forebrain cholinergic neurons to Aβ [26]. Intracerebroventricular injection of Aβ induced reduction in ChAT activity in the medial septum and hippocampus [53]. We observed not only Aβ- or CT105-induced reduction of ACh release in the hippocampus but also CT105-induced reduction of ChAT immunoreactive neurons in the hippocampal area. Interestingly, CT105 reduced ACh release more severely than Aβ in the hippocampus at

1 week after CT105 treatment, supporting previous evidence that CT-induced neurotoxicity is stronger than Aβ-induced toxicity [49]. The number of ChAT positive neurons and ACh release are reduced by CT105 may implicate the loss of cholinergic function including ACh synthesis via ChAT.

The present study shows that the number of ChAT positive neurons are reduced in the vertical and horizontal limbs of the diagonal band of the CT105-injected rats. Since the diagonal band is well known as a main route for the cholinergic innervation to the hippocampus [17, 32, 39], neuronal loss in this area affect learning and memory. In fact, some previous data consistently have supported a close relationship between neuronal damages in the diagonal band and the cognitive impairments. Excitotoxic or pharmacological lesions of the vertical diagonal band of Broca produce deficits in hippocampal cholinergic neurons and learning performance [13, 36] in animal model. Therefore, our finding indicates that the cholinergic neuronal loss induced by CT105 in the diagonal band of Broca may contribute to the cognitive processes directly. Rats received intraventrical injection of CT105 displayed significant cholinergic neuronal loss in the medial septum. Although it was checked in different animal model from our animal model, a lot of previous pharmacological or pathological data have supported that medial septal region also critically involved in learning and memory. Selective lesion of chlolinergic neurons in the medial septum impairs acquisition of a delayed matching-to-position spatial memory [24, 25]. GABA-ergic compounds into the medial septum impairs performance in memory-related behavioral tasks, in particular, those associated spatial learning [9, 12]. Electrolytic medial septal-lesioned mice show the spatial learning deficits [22]. Therefore, CT105-induced ChAT positive neuronal loss in the medial septum might lead to cognitive dysfunction associated with learning and memory impairments [7].

It is postulated that the decrease in ChAT activity in the medial septum and the diagonal band of Broca of CT-injected rat show abnormality in septo-hippocampal pathway contributing learning and memory. Cholinergic and parvalbumin-containing GABA-ergic medial septum diagonal band neurons project to the hippocampal formation. Cholinergic neurons represent roughly two thirds (65%) of the total projection neurons, whereas parvalbumin-containing GABA-ergic cells represent one third. Therefore, they account for nearly all of the septo-hippocampal projection neurons [45]. The CT105-induced decrease of ACh release determined by microdialysis method provides indirect evidence that CT105 can affect the release of ACh into synaptic cleft or ACh synthesis in the presynapse. Previous report showed that the decrease in total ACh level in the cortex and hippocampus via the reduction of pyruvate dehydrogenase activity induced by CT105 in CT105-injected mice model [7]. Extracellular ACh release in the hippocampus is positively related to the activity of septohippocampal cholinergic neuron functioning to spatial memory performance [14]. The full destruction of the cholinergic septohippocampal pathway decrease hippocampal ChAT activity, ACh synthesize and ACh release *in vitro* and *in vivo* model, although following partial destruction of afferent cholinergic fibers that innervate the hippocampal formation can be compensated by residual cholinergic neuron [34]. Previous study observed that impaired septohippocampal pathway decreased ChAT protein expression in hippocampus and medial septal neurons [19]. APP transgenic mice (London mutation) showed cholinergic deafferentation and cholinergic cell shrinkage [6], providing direct evidence that C-terminal fragment of APP may contribute to cholinergic degeneration observed in our present study.

Taken together, the results in the present study suggest that CT105 impairs the septohippocampal pathway by reducing acetylcholine synthesis and release, which results in damage of learning and memory.

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