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Effects of Bombusae concretio Salicea on Amyloid-ß-induced Neuronal Cell Toxicity and Lipid Peroxidation in Cultured Rat Astrocytes

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive decline resulting from selective neuronal dysfunction, synaptic loss, and neuronal cell death. The well-studied neuropathological features of AD showed the following characters such as loss of neurons, formation of intra-neuronal neurofibrillary tangles composed of paired helical filaments of the cytoskeletal protein tau, and extracellular plaques composed primarily of diffuse or compacted deposits of amyloid-ß (Aß) aggregates, with or without a component of dystrophic neurities [1,2].

It has been suggested that glial cells in AD may play in the neurodegenerative cascade and leads to Alzheimer dementia. Neurodegerative disorders usually involves the activation of astrocytes and gliosis (microglia). Glial activation involves morphological changes (more spherical cell soma, hypertrophy of nuclei, appearance of extensive cellular processes) and changes in expression of a large number of proteins [3]. In AD, activated astrocytes surround the neuritic shell of the amyloid plaque, and activated microglia are near the center of the neuritic shell sdjacent to the amyloid core [4]. There are a number of stimuli that cause glial activation. One of the the best inducers of glial activation is neuronal dysfunction or injury. Although the role of glial activation in AD is uncertain, it was known that reactive glia are associated with amyloid plaques [5]. In addition, some cytokines and inflammatory mediators produced by activated glia have the potential to initiate do excerbate the progression of neuropathology [5,6,7,8].

The factors responsible for inducing and maintaining the glial activation state in AD are unknown. However, it was suggested that Aß is involved in the neurodegenerative process. Aß is 39-43 amino acids long and proteolytically derived from an integral membrane protein termed amyloid precursor protein (APP) [9,10], although mechanism for APP processing is not still unknown. There are many *in vitro* studies demonstrating that Aß is directly neurotoxic and increase

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neuronal susceptibility to other toxic agents [5,10,11]. The toxic effect of Aß is correlated with its ability to form aggregates [12]. Both oxygen species [13] and excessive Ca^{2+} influx [14] are also implicated in the mechanism of Aß neurotoxicity. In contrast, it was also reported that Aß promotes neurite outgrowth under certain culture conditions instead of toxic action [15].

On the other hand, the action of Aß that affect glia, and the glial responses to Aß exposure are not well understood. When the effects of synthesis Aß peptides(Aß1-42, Aß17-42, and scrambled Aß1-42) on cultured astrocytes were examined, Aß1-42 induced a robust astrocyte activation, as evidenced by morphological changes, upregulation of the interleukin-1ßmRNA, and stimulation of inducible nitric oxide synthase (iNOS) mRNA and nitric oxide (NO) release [16].

The reactivity of different free radicals varies and some cause severe damage to biological molecules, especially to DNA, lipids and proteins. In the presence of oxygen, free radicals can react with polyunsaturated fatty acids, resulting in highly reactive peroxyl free radicals. Peroxyl free radicals can further propagate the peroxidation of lipids or compromise the integrity of cell membranes, therefore this is thought to be involved toxic actions of some chemicals [17-19].

Recently, several reports presented that natural dietary plants may play an antioxidative role in the prevention of aging and carcinogenesis and may offer effective protection from lipid peroxidative damage *in vitro* and *in vivo* [20,21]. Therefore, much attention has been focused on natural antioxidants, in paticular it was reported the extract of *Bombusae concretio Silicea* (BCS) being used for cerebrovascular accident and aphasia by the activites of treatment on Wind-heat syndrome and heat-phlegm in oriental medicine[22], although little is yet known about the pharmacological effects or active ingredients.

As an approach to know the interactions between Aß and glia, we have further examined the effects of synthesis Aß peptides on cultured astrocytes. We tested Aß25-35 peptides aggregated under various conditions. We report here that Aß25-35 is responsible for the activaion of the astrocytes. This study also reports the effect of BCS on cytotoxicity of cultured astrocytes and lipid peroxidation in Aß-treated conditions. Aßwhich can produce intracellular free radical was used for inducer of the peroxidation of cellular lipids.

MATERIALS AND METHODS

Materials

The Aß25-35 peptide was synthesized by Applied Biosystem's Protein Synthesizer Model 470A (Peptron Co., LTD, Taejon, Korea). Fetal bovine serum (FBS), penicillin- streptomycin were obtained from GIBCO-BRL (Grand Island, New York, USA). Dulbecco's Modified Eagle's Medium (DMEM), glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasolium bromide(MTT), dimethyl sulphoxide (DMSO), 2-thiobabituric acid (TBA), 1,1,3,3-tetraethoxypropane(TEP), ethylenediamine

tetrazolium (NBT), catalase (from bovine liver), diethylene triamine pentaacetic acid (DETAPAC), β-nicotinamide adenine dinucleotide phosphate (β-NADPH) were purchased from Sigma Chem. Co. (St. Louis, USA). 1-chloro-2,4-dinitrobenzene (CDNB), NaN_3 were obtain from Aldrich Chem. Co. (Milwaukee, WI).

Extract from Bombusae concretio Salicea (BCS)

Bombusae concretio Salicea (300 g) purchased from herbal store and extracted with boiling water for 3 h. Then, the extract was evaporated to under reduced pressure by 75%, 85%, 95% ethanol solution. The last extracts diluted by 0.9% NaCl and filterated. The extract solution was stored at 4℃

Cell culture and preparation of Bombusae concretio Salicea

Cortical astrocyte cultures were prepared from neonatal rat (1-2 day old) pups by the method of Levision and McCarthy [23]. Cerebral cortex was dissected from neonatal day 1-2 Sprague-Dawley rat and dissociated by gentle trituration. Cells were plated in 6-well culture plates coated with polyethylenimine (0.2 mg/ml in sodium borate buffer, pH 8.3) at a density of 40,000 cells per well. After overnight incubation in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 20% fetal bovine serum, the medium was changed to serum-free defined medium for neurons [DMEM supplemented with 2 mM glutamine, 1 mM pyruvate, penicillin-streptomycin-amphotericin B mixture (Gibco), 5 mM HEPES, 0.5% glucose, 10 μg/ml insulin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, and 20 μg/ml transferrin]. The cultures were incubated at 37℃ in an atmosphere of 5% CO_2/95% room air, and the medium was replaced every other day. Experiments were performed in 6-7-day-old culture.

Depending upon the experimental group, BCS was added (at 2% volume in culture medium) to or omitted from flasks. After 16~18 h, cells were washed twice with warm phosphate-buffered saline (PBS) and serum-free medium added to the flask. Then the cells were treated with 10 μM Aß peptide for 2 h and the content of thiobarbituric acid-reactive substance (TBARS) and enzyme activities measured. 10 μM Aß peptide was diluted in serum-free medium and added to the cultures.

Determination of cell viability and toxicity assay

Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the coloured formazan product by mitochondrial enzymes in viable cells [24]. Cell were cultured in polyethylenimine-coated 24 or 96 well culture plates at a density of 10,000 cells per well for lactate dehydrogenase (LDH) assay or 40,000 cells per well doe MTT reduction assay. LDH activities in the medium were measured by a Cytotox 96 nonradioactive cytotoxicity assay kit (Promega) according to the manufacturer's instructions. The results were expressed as percentages of peak LDH release on complete cell lysis

(control).

The MTT reduction was measured essentially as described previously [10] with a slight modification. In brief, after incubating cells for 48 h with various samples, *t*-BHP and Aß peptides, MTT (Sigma) solution in PBS was added to a final concentration of 0.5 mg/ml, and the cells were further incubated for 4 h at 37℃. After incubation, the plate were centrifuged at 90 x *g* for 10 min to obtain the resulting insoluble formazan precipitates. To dissolve the crystal precipitates, 150 μl or 600 μl of a 1:1 mix of ethanol and DMSO were added to each well. Each plate was gently shaken for approximately 20 min before reading on the Enzyme-Linked Immunosorbent Assay (ELISA) reader (measurement 570 nm, reference 620 nm). Absorbance of converted dye was measured. Assay values obtained on addition of vehicle were taken as 100%, and complete inhibition of MTT reduction (0%) was defined as the value obtained following addition of 0.9% Triton X-100.

To examine whether BCS could attenuate the cytotoxicity of Aß peptides, cultures were pretreated with indicated concentrations of BCS for 4 h. Thereafter either 10 μM Aß 25-35 was added to cultures and incubated for 48 h. LDH activity in the culture medium was determined as described above. To investigate the effect of pretreatment with Aß peptides on the cytotoxicity induced by hydrogen peroxide or glutamate, cells were pretreated with 10 μM Aß peptide for 48 h, and then 100 μM hydrogen peroxide or 100 μM glutamate was added to cultures and incubated further for 4 h or 1 h, respectively.For all findings, each condition represents five separate wells pwe experiment and is repeated in two or five independent experments.

Treatment of astrocytes with Aß peptides

Confluent astrocytes were trypsinized and plated into T-75 tissue culture flasks at a density of 5 x 10^6 cells/flasks (for lipid peroxidation and antioxidative enzyme activity), or into 96-well plates at a density of 5 x 10^4 cells/well (for MTT reduction assays). After 24 h, cells were washed with PBS to remove serum, and cultures were incubated in DMEM free FBS for an additional 12 h before addition of Aß peptides or control buffer.

Lipid peroxidation assay

The release of TBARS into incubation medium was measured by the method of Glascott *et al.* [25]. Depending upon the experimental group, BCS was added (at 2% volume in culture medium) to or omitted from flasks and then overnight incubation. Overnight (16~18 hr) cells were washed twice with warm PBS buffer and 10 ml of serum-free medium were added to the flask, and the cells were treated with Aß peptide. After Aß peptide treatment for 2 hr, the cells were removed by scraping, then trichloroacetic acid (TCA) was added to scraped cell and medium (4.5% final concentration). The scraped cells were sonicated for 20 sec and centrifuged to pellet the protein. In brief, 1 ml of TCA supernatant was added to 2 ml of TBA solution (composed of 0.45%, w/v, TBA and

7.5%, v/v, acetic acid, pH to 4.15 with 10 N NaOH). This reactive solution was placed in a boiling water bath for 15 min, cooled to room temperature and read on a Gilford Response spectrophotometer with excitation wavelength of 532 nm. TEP was dissolved in 0.01 N HCl to produce malondialdehyde (MDA), and this was used to generate a TBARS standard curve. The data was expressed as nmol of TBARS/mg protein

Protein determination

Protein was determined on each sample by the method of Smith *et al.* [26] (using bicinchoninic acid), using bovine serum albumine as the standard.

Statistical analysis

Standard procedures were use to calculate means and standard deviation of the mean. Mean values were compared using Duncan′ s Multiple Range Test with on SAS program (SAS Institute, Cary, NC) ; $P<0.05$ was considered significant.

RESULTS & DISCUSSION

Effect of amyloidß25-35 peptide on cell killing in cultured astrocyte cells

As shown in Fig. 1, cell killing was significantly enhanced by addition of increasing concentrations of Aß compared to those of untreated group. When we measured the amount of MTT reduction from cultured rat cortical astrocyte cells at 48 h after peptide treatment, the Aß25-35 decreased MTT reduction by 55% of the control value at 0.1 μM concentration. Aß25-35 reduced MTT reduction by 30% and 20% at 50 μM and 100 μM, respectively.

On the other hand, the toxicities of Aß25-35 were assessed by LDH assay. Following the appropriate incubation time with various peptides, LDH activities in the medium were measured by a Cytotox96 nonradioactive cytotoxicity assay kit (Promega) according to the manufacturer's guidances. The results were expressed as percentage of peak LDH release obtained on complete lysis. The Aß25-35 peptide increased LDH ·release by 52.63% of themaximal value at 100 μM concentration. Aß25-35 induced LDH release only 12.3 and 32.5%, respectively, even at 25 and 50 μM (Table 1). However, non-toxic fragment of Aß, had little effect on LDH release up to 100 μM.

Furthermore, we measured the protective and proliferative effects of BCS on LDH activity in 24 h before treatment with indicated concentrations of Aß25-35. The treatment of 100 μg/ml of concentrated BCS solution (10-times diluted from 1000 μg/ml in Table 1) reduced the LDH activity by 75% of control group when compared at concentration of 100 μM Aß25-35 (52.63 vs 39.35). Also, when the protective and proliferative effects of BCS on LDH activity in 48 h after treatment with indicated concentrations of Aß25-35, the treatment (100 μg/ml) of BCS solution reduced the LDH activity by 90% of control group of 100 μM Aß25-35 (52.63 vs 47.47). This

is much more effective for astrocyte protection than posttreatment of BCS.

Effect of Bombusae concretio Salicea (BCS) on cell killing in cultured astrocyte cells

We measured the protective and proliferative effects of BCS on MTT reduction in cultured astrocytes at 48 h after BCS treatment (Fig. 2). The treatment of 100 μg/ml of concentrated BCS solution (10-times diluted from 1000 μg/ml in Fig. 2) increased the MTT reduction activity by 140% of control group. Interestingly, 20 μg/ml (50-times diluted) and 10 μg/ml (100-times diluted) concentrations of the BCS solution resulted in by 165% and 370% increase of the control group, respectively, being maximal MTT reduction activity at 10 μg/ml. Upon further dilution of the BCS solution up to 0.1 μg/ml concentration, the reduction activity was higher than that of vehicle. Thus, it was possibly concluded that the BCS is highly effective for the protection and proliferation of the rat cortical astrocytes.

On the other hand, pretreatment of BCS attenuated in a cell killing enhanced by exposed to increasing concentrations of Aß25-35 (Fig. 3). This indicates that cells pretreated with BCS allowed its resistance against the toxic effects of increasing concentrations of Aß25-35 peptides. However, gradual dilution of the BCS concentration increased cell killing activity of Aß25-35. With the pretreatment with 1000-times diluted BCS, about 70% of the cells were killed within 48 h by 50 μM Aß25-35. In contrast, fewer than 60% of the 100-times diluted BCS pretreated cells were killed by the same concentrations of Aß25-35. Seemingly, post-treatment with serially diluted BCS showed similar cell killing activity in the with or without BCS pretreatment (Fig. 4).

Effect of Bombusae concretio Salicea (BCS) on lipid peroxidation induced by Aß25-35 in cultured astrocyte cells

Fig. 5 shows the effect of BCS on MDA level in cultured rat astrocyte cell exposed to Aß25-35. The accumulation of MDA, as measured by TBARS in the medium, is a sensitive index of the peroxidation of cellular lipids in cultured cell intoxicated with Aß25-35. At present assay, TBARS levels of Aß25-35 treatment group were significantly higher than other groups. This increased level was significantly reduced by BCS pretreatment. These results of cell killing and MDA level by Aß25-35 treatment are in agreement with those of Glascott *et al.* [25,27]. In addition, lipid peroxidation was prevented or greatly reduced by addition of antioxidants (Vit E, Vit C, DPPD or deferoxamine) [28]. For example, addition of antioxidants in cell culture medium significantly reduced cell killing and content of intracellular antioxidants. And, in the case of BCS+Aß25-35 group, MDA level induced by Aß25-35 was significantly reduced by BCS pretreatment.

Concentration (μ)	LDH(% of maximal release)		
	Without	Pretreatment of BCS(100㎍/㎖)	Posttreatment of BCS(100㎍/㎖)
0.1	2.65±0.18	2.84±0.23	2.77±0.19
0.5	2.67±0.23	2.54±0.23	2.86±0.21
1.0	3.23±0.36	3.32±0.31	4.44±0.34
5.0	4.05±0.38	3.65±0.29	3.81±0.36
10	6.43±0.72	5.21±0.45	6.12±0.56
25	12.3±1.43	7.43±1.11	9.75±1.08
50	32.5±4.13	18.73±2.43	29.33±3.13
100	52.63±6.21	39.35±4.65	47.47±4.23

Table 1. LDH activity in the culture medium of cultured rat cortical astrocytes at 48 h after treatment with indicated concentrations of Aβ25-35 and protective effects of BCS treatment.
The results are expressed as percentage of maximal LDH release that was obtained on complete cell lysis. Data are mean ± SEM values obtained from five culture wells per experiment, determined in three to five independent experiments. *, P<0.05. **, P<0.001.

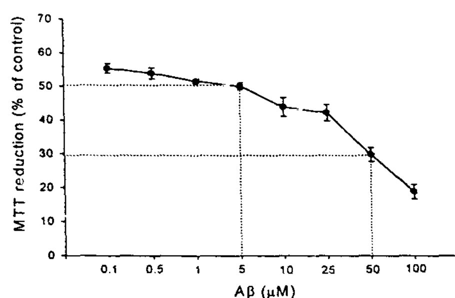

Fig. 1. Effects of various concentrations of Aβ25-35 on MTT reduction in cultured rat cortical astrocytes.
Cell were incubated with the indicated concentrations of Aβ25-35 for 48 h. Assay values obtained on addition of vehicle were taken as 100% and complete inhibition of MTT reduction (0%) was defined as the assay value obtained following addition of 0.9% Tritone X-100 to lyse the cells completely.

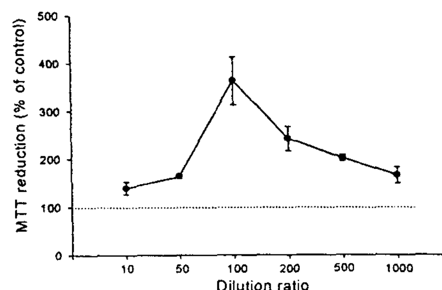

Fig. 2. Effects of various concentrations of BCS on MTT reduction in cultured rat cortical astrocytes.

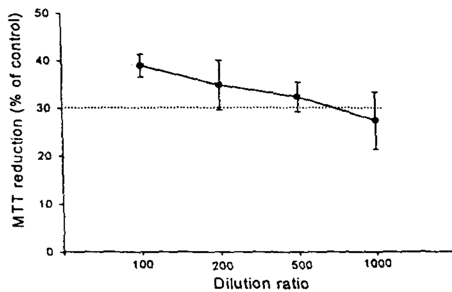

Fig. 3. Effects of pretreatment of BCS on Aß25-35-induced cytotoxicity in cultured rat cortical astrocytes. Cultures were pretreated with various concentrations of BCS for 24 h before application of 50 μM Aß25-35. At 48 h after 50 μM Aß25-35 treatments, MTT reduction was assayed.

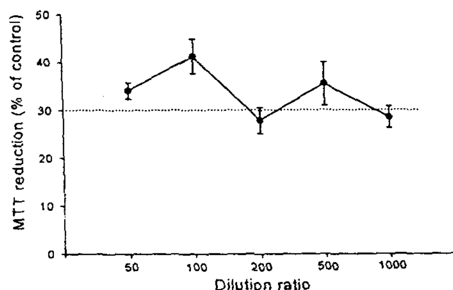

Fig. 4. Effects of BCS on Aß25-35-induced cytotoxicity of cultured rat cortical astrocytes. Cultures were treated with 50 μM Aß25-35 for 48 h before application of various concentrations of BCS. At 24 h after BCS treatment, MTT reduction was assayed.

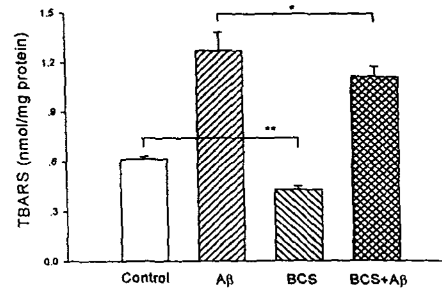

Fig. 5. Effects of BCS on lipid peroxidation induced by Aß25~35 treatment in cultured rat cortical astrocytes. Culture cell were pretreated with BCS for 24 h before application of 50 μM Aß25-35. After 48 h, the release of TBARS into incubation medium was measured. * $P<0.05$, ** $P<0.001$.

SUMMARY

The present study was done to investigate the effects of *Bombusae concretio Salicea* (BCS) on cultured astrocyte cell system and lipid peroxidation in Aß25-35 treatment conditions. Cell killing was significantly enhanced by addition of increasing concentrations of Aß25-35. Pretreatment of BCS attenuated in cell killing enhanced by increasing concentrations of Aß25-35. MDA level induced by Aß25-35 treatment was significantly increased and the level was slightly reduced by pretreatment of BCS. The present study showed that Aß25-35 strongly increased MDA level and the level was enhanced by addition of increasing concentrations of

Aß25-35 or by time-related exposure to Aß25-35.

In conclusion, it was shown that Aß25-35 is not only potent lipid peroxide inducer, but also cause protection of neurodegeneration induced by Aß25-35. It can be concluded that the activation of antioxidative enzymes may be related to the inhibition of lipid peroxidative reactions. We cannot fully explain to effects of BCS at present; however, the ability of BCS to reduce cell killing and MDA level induced by Aß25-35 suggest that BCS may be a protective agent for free radical generating compounds such as Aß25-35.

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국 문 초 록

흰쥐 astrocyte에 있어서 amyloid-ß에 의한 독성과 지질과산화에 미치는 天竺黃의 영향

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天竺黃은 한의학에서 淸風熱과 治痰하는 효능으로 중풍과 不語症을 치료하는데 널리 사용되고 있다. 본 연구에서는 天竺黃이 실험적인 치매(Alzheimer Disease; AD)를 유발시키는 물질로 알려진 amyloid-ß (Aß peptide)를 흰쥐의 신경세포의 일종인 astrocyte에 처리하여 그 세포독성과 보호효과 및 세포막 지질의 과산화에 미치는 영향을 검토하였다. 天竺黃은 Aß에 의한 신경세포에 대한 손상을 억제하여 세포증식을 촉진하여 예방 및 보호효과를 나타내었다. 또한, 세포막 지질의 과산화의 지표인 malondialdehyde (MDA)생성이 Aß처리로 크게 증가하였으나 세포막 파괴에 의한 뇌세포 파괴의 전형적인 현상이 天竺黃의 前처리와 後처리로 크게 감소되었다. 그리고, 이러한 결과들은 天竺黃이 신경세포의 하나인 astrocyte에 대한 보호효과와 세포막지질의 과산화 저해 및 Aß처리와 같은 치매유발 독성에 대한 적응능력 향상을 통한 뇌신경의 보호효과를 주는 것으로 노인성 치매 등 임상적 응용에 그 효과가 기대된다.

Key Words : *Bombusae concretio Salicea,* dementia, amyloid-ß, astrocytes, lipid peroxidation