Anti-inflammatory Effect of Bear's Gall in Rat Microglia

Seong Soo Joo, Yeong Min Yoo[1], Seon Goo Lee[1]*, Do Ik Lee

Department of Immunology, College of Pharmacy, Chung-Ang University,
1: Department of Oriental Pathology, College of Oriental Medicine, Sangji University

We hypothesize that bear's gall may have a certain role in anti-inflammation through a preventive effect of pro-inflammatory potentials. Secondly, we tried to connect the experimental results to Alzheimer's disease (AD), which chronic inflammation is a main cause of the disease. For this theme, we designed to elucidate the efficacy of bear's gall in suppressing the pro-inflammatory mediators, such as nitric oxide (NO) and interleukin-1β (IL-1β) in rat microglia. From the study, we concluded that bear's gall plays a positive role in suppressing such pro-inflammatory repertoire from rat microglia comparing to normal and positive control, such as culture media and cyclosporine. Interestingly, bear's gall showed a prolonged effect of anti-inflammation comparing with cyclosporine when time goes by up to 48h with a significant suppression at 1.2 ㎎/㎖. Therefore, we can consider that bear's gall in part can be applied to AD therapy in that it suppresses the expression of pro-inflammatory mediators as well as its continued effect.

Key words : microglia, bear's gall, Alzheimer's disease, IL-1β, Nitric Oxide

Introduction

Alzheimer's disease(AD) is a neurodegenerative disease and also known for the most common form of dementia is first reported by Dr. A. Alzheimer in 1907. In patient with AD, senile plaques(SP) and neurofibrilar tangles(NFT) are a common founding and those founding are reported to play a major role in the disease progression[1-3]. β-amyloid peptide(Aβ) produced from transmembraneous amyloid precursor protein(APP) and microtubule associated protein(tau protein) are known as the major causative factors of AD[4]. The Aβ peptides from APP by dual cleavage action of β & λ secretase stimulate the brain glial cells (i.e. microglia) to release pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, and nitric oxide, which lead to chronic inflammation in the brain by an escalation of reactive oxygen species(ROS), pro-inflammatory cytokines, and neurotoxic factors[5-7]. On the other hand, the Aβ directly induces cytochrome c release from mitochondria through a mechanism that is accompanied by profound effects on mitochondrial membrane redox status, lipid polarity, and protein order.

In this relation, main components of bear's gall, ursodeoxycholic acid and tauroursodeoxycholate, can directly suppress Aβ-induced disruption of the mitochondrial membrane structure, suggesting a neuroprotective role for this bile salt[8]. In addition, as the Aβ accumulated intracellularly by APP processing might exert neurotoxicity by interacting with mitochondria and inducing mitochondrial swelling and release of Cyt c, which activates caspase-3 and finally can lead to apoptosis in neuronal cells and to neurodegeneration in AD[9], it will be a meaningful concept in AD therapy if ursodeoxycholic acid as reported previously plays a role in suppressing the mitochondrial damage by way of oxidative stresses as well as chronic inflammation by cytokines.

The hypothesis adapted in this study was "Amyloid Hypothesis" that has been adopted in tens of AD researches for the last decades and that has been centered in molecular approaches of AD-related studies. In short, "Amyloid (cascade) Hypothesis" is based on the neurotoxic properties of Aβ[10], and posits that increased secretion of Aβ leads to elevated extracellular levels of Aβ as senile plaques, which in turn are toxic to surrounding neurons[11]. Therefore, to prevent such plaques formed from Aβ peptides, fast and effective clearance of Aβ peptides should be done, or if the plaques are already formed, the attenuation of microglial activation should be considered in AD therapy. When microglia are activated, they rapidly produce pro-inflammatory mediators and cause a chronic inflammation. Virtually, Aβ is a good activator to microglia and is known to play a role to aggregate the disease

* To whom correspondence should be addressed at : Seon-Goo Lee, Department of Oriental Pathology, College of Oriental Medicine, Sangji University

· E-mail : returnto@sangji.ac.kr, · Tel : 033-730-0664

· Received : 2004/11/25 · Revised : 2004/12/23 · Accepted : 2005/01/26

states in AD patients. In other words, a massive production of Aβ peptides from APP cause neuritic plaques and activated microglia by fibrillar Aβ(fAβ) are surrounded the vicinity of plaques. In this state, microglia release proinflammatory cytokine such as IL-1β[7] and nitric oxide[12] and thus lead to chronic inflammation. With this background, we expected that the attenuation of microglial activation would be a key impact on in-progress AD therapy.

On the other hand, ursodeoxycholic acid that was first isolated by Hammersten in 1902 from the bear bile acid has known to inhibit the expression of IL-2, IL-4, and IFN-γ in T-cells as well as immunoglobulin in B-cells[13-15]. In addition, ursodeoxycholic acid may inhibit the macrophagic activities such as ROS, NO, and IL-1β from kupffer cells stimulated by hydrophobic bile acid[14,16]. In this study, we wanted to elucidate the anti-inflammatory effect of bear's gall in microglia at the level of the rat brain by screening the NO and cytokine expression (IL-1β) when stimulating microglia with A β42, cyclosporine, and the culture media.

Methods and Materials

1. Test animal

The study was performed by using 1-day old Sprague Dawley(SD) rat.

2. Test drug

Bear's gall gifted by Oriental Pharmaceutical Company. Bear's gall was dissolved in 95% EtOH and prepared by mixing with 5% FBS-MEM (culture media). The screened concentrations were ranged from 0.8 ㎎/㎖ to 0.8 g/㎖ for multiple time courses appropriated for the study interests.

3. Reagent

Cyclosporine A (Sigma Chem., U.S.A.), EtOH (Merck, Germany), FBS (Gibco, U.S.A.), Griess Reagent (Promega, U.S.A.), HEPES (Duchefa Biochem, Netherlands), L-glutamine (Sigma, U.S.A.), Lipopolysaccharide (E. coli, serotype 0111:B4) (Sigma Chem., U.S.A.), Penicillin-Streptomycin (Gibco, U.S.A.), Rat IL-1β & TNF-α EIA Kits (Assay Designs, U.S.A.), Trypsin (Gibco, U.S.A.), Aβ42 (Tocris, U.S.A.) were used. In addition, other buffers and reagents used in the study were formulated in lab with the first or super graded reagents.

4. Tools and equipments

ELISA Reader (Bio-Rad, Japan), Water bath (Vision Sci., Korea), Clean bench (Vision Sci Co., Korea), CO_2-incubator (Sanyo Electric Co., Japan), centrifuge (Vision Sci. Co., Korea), microcentrifuge (Vision Sci. Co., Korea), deep freezer (Sanyo Electric Co., Japan), inverted microscope (Olympus Optical Co., Japan), electrophoresis (Vision Sci. Co., Korea), vortex mixer (Scientific Industries INC., U.S.A.), thermal cycler (Barnstead Thermolyne Corporation., U.S.A.), electronic balance (A&D Co., Japan), UV-VIS spectrophotometer (Shimadzu Corporation., Japan), polaroid type 667 film (Polaroid Co., United Kingdom), pH meter (Mettler toledo, Switzerland), autoclave (Vision sci. co., Korea). All other tools and equipments used during the study were autoclaved at 121℃, 1.1㎏/㎠ for 20 minutes before use.

5. Isolation of Microglia

Microglial cells for primary culture were isolated from mixed glia prepared from newborn Sprague-Dawley rats (1-day-old, SD rats) in accordance with the lab protocol, which has been modified from previous reports[17]. To prepare primary cultures of mixed glia, we obtained neocortical tissues of newborn SD rats, removed the meninges, minced well with micro tips and incubated the tissues in 0.05% trypsin (Gibco) for 15 minutes. After incubation, the tissue suspension was centrifuged for 7 minutes at 1450 rpm and resuspended in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc) with no serum for the titration of primarily minced tissues with finely fire-polished, Pasteur pipettes by diminishing the tip holes to one forth in diameter. The supernatant was then centrifuged once more and the pellets resuspended with DMEM supplemented with 10% heat-activated fetal bovine serum (FBS), 100 units/㎖ penicillin and 100 ㎎/㎖ streptomycin (10% FBS-DMEM) (Gibco). Cells were then grown in 75 cm^2 culture flask (1.5 cortices per flask) containing 10 ㎖ of 10% FBS-DMEM at 37℃ in a 5% CO_2 humidified air atmosphere for 2 weeks. After the primary culture (1 week later), the microglia were detached from the flask by tapping softly the side edges several times with mild fluctuation of the media and filtered by passing though a 33 ㎛ nylon mesh to remove astrocytes and clumped unnecessary cells. The supernatant was centrifuged at 1450 rpm and then microglia were resuspended and replaced in 25 cm^2 culture flask containing 5% FBS-DMEM at a density of about 2.0×10^7. The cultures were shaken to release microglia twice a week for 2 to 3 weeks in accordance with the study designs. In principal, we kept the cell density at 1.0×10^5 per experimental well. Cell purity was confirmed by DiI-LDL uptake of microglia under fluorescence microscopy (>98%).

6. NO Assay

Nitric oxide was detected as indicated in the company's

manual insert. In brief, we prepared a nitrite standard solution ranged from 1.56 μM to 100 μM for a standard reference curve. For the nitrite measurement (Griess reaction), we added 50 μℓ of each experimental samples to wells in triplicate, and then dispensed 50 μℓ of the Sulfanilamide Solution to all experimental samples and wells containing the dilution series for the Nitrite Standard reference curve. We then incubated 10 minutes at room temperature, protected from light and dispensed 50 μℓ of the NED (N-1-napthylethylenediamine dihydrochloride) Solution to all wells. Following 10 minutes at room temperature by protecting from light, we measured absorbance within 30 minutes in a plate reader with a filter 540 ㎚.

7. RT-PCR (Reverse Transcriptase Polymerase Chain Reaction)

Total RNA was extracted using the TRIZOL method (Molecular Research Center). 2 μg of total cellular RNA were reverse transcribed for 20 μℓ reaction using RT Premix (AccuPower RT Premix, Bioneer, Korea), which containing Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT), RNAse inhibitor, and others specific for synthesizing cDNA, according to the manufacturer's instruction. In brief, the template RNA was mixed with RNA specific primers and incubated the mixture at 70℃ for 5 minutes and place it on ice. The incubated mixture was transferred to RT Premix following the fill-up of the reaction volume with DEPC-DW. RT tubes were then dissolved well by vortexing and briefly spined down by mini-centrifuge. cDNA synthesis reaction was performed at 42 ℃ for 60 minutes for cDNA synthesis and at 94 ℃ for 5 minutes for RTase inactivation. After the reaction, RT tubes were placed on ice, immediately.

PCR reaction was carried out in a AmplitronIII® Thermal cycler (Barnstead Thermolyne Corp. U.S.A.) with 35 cycles of 95℃ for 5 min (pre-dwell), 95℃ for 30sec, 60℃ for 60 sec, 72℃ for 60sec, followed by 72℃ for 10min, then held at 4℃. PCR reactions were in a final volume of 20 μℓ of the following reaction mixture: PCR buffer (10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, pH 9.0) 250 μM dNTP, 1 unit Taq DNA polymerase, 2μℓ of cDNA for amplification of IL-1β, and β-actin. In all experiments, β-actin was served as an internal control. The used primer sequences were as follows: for IL-1β, 5′-GAA GCT GTG GCA GCT ACC TAT GTC T-3′ and 5′-CTC TGC TTG AGA GGT GCT GAT GTA C-3′ (520 bp target size); for β-actin, 5′-GTG GGG CGC CCC AGG CAC CA-3′ and 5′-GTC CTT AAT GTC ACG CAC GAT TTC-3′ (526 bp target size) based on the published DNA sequence[18]. Reaction products were separated by electrophoresis on a 2.0% agarose gel containing ethidium bromide (Sigma, U.S.A.) in buffer (40 mM Tris-acetate and 1 mM EDTA, pH 9.0), visualized in U.V. plate, photographed and transferred to computer using digital camera (Sony, Japan).

8. Cytokine EIAs (Enzyme Immunometric Assay)

EIA kits (TiterZyme® EIA, Assay Designs, U.S.A.) were used for the quantitative determination of level of the cytokines (IL-1β) produced from microglia in response to stimuli of positive or negative substances. The kit is an assay tool designed to use polyclonal antibody to rat cytokines immobilized on a microtiter plate to bind the rat cytokines in the sample. This was carried out in accordance with the manufacturer's protocol and read the optical density of the plate at 450 ㎚ by the plate reader (Bio-RAD, Japan). In brief, cultured plates were taken into clean bench and 100 μℓ of samples were pipetted into the wells of provided microplate, tapped the plate gently to mix the contents, and sealed with the plate sealer. We then incubated it at 37℃ for 1 hour. After incubation, the plate was washed several times by adding 200 μℓ of wash solution with multichannel pipette, and pippetted 100 μℓ of the labeled antibody into each well, and sealed again the plate and incubated at 4℃ for 30 min. The plate was washed entirely by adding 200 μℓ with wash solution and 100 μℓ of the substrate solution was added to each well flowed by 30 min. of incubation at room temperature in the dark environment. After the incubation time, the reaction was completed by adding 100 μℓ of stop solution. The concentration of cytokines was represented in pg/㎖ through the calculation from the average OD and standard curve.

Results

1. NO Assay

To investigate the NO production in various time courses, we set the bear's gall concentration from 200 μM to 2 mM and studies were designed in three separate groups, culture media, LPS, and Aβ42 for 6, 12, 24, and 48hours (Fig. 1, 2, 3). In pre-study (data not shown), we found that microglia in 5% FBS culture media produced small amount of NO from 6 to 24hour in a constant manner, but it was not detectable in microplate reader after 48hour. Therefore, we ignored the NO from microglia in non-treated group (normal control). Cyclosporine showed to effectively suppress the NO production at 12h, while it relatively increased after 24h (Fig. 1). Contrarily, LPS group activated microglia and induced the NO production up to 12h, but the NO was decreased when reached 48h. As expected, the positive control, cyclosporine, suppressed more effectively than that of bear's gall in earlier time phase. When compared these results with LPS, an

activator for the production of NO from microglia, bear's gall is also considered to suppress in significant level. In addition, we found that bear's gall lasted its effectiveness during the whole chased times even though the level was slightly increased. The intervals between the pre- & -post time point in bear's gall group were less than that of cyclosporine (Fig. 2). When treating with Aβ42, NO was significantly increased at 24 and 48h. This reveals that Aβ42 activates microglia in time dependant manner, and both bear's gall and cyclosporine in combination with Aβ42 were constantly posited under the normal control up to 24h although there was non-significant upregulation of NO at 48h in both (Fig. 3). The single treatment of bear's gall showed the lower level of NO than control group in 0.8 ㎎/㎖ and 1.2 ㎎/㎖. However, the highest concentration, 0.8 g/㎖ did not show the dramatic result comparing to 1.2 ㎎/㎖ bear's gall, which means that it is no dose-dependent. The NO levels in Aβ42 group showed to be upregulated comparing to the normal control, but the combination with bear's gall reduced NO in 0.8 ㎎/㎖ and significantly in 1.2 ㎎/㎖. However, no more reduction of NO was observed in higher concentration, 2.0 ㎎/㎖ and 0.8 g/㎖ (data not shown).

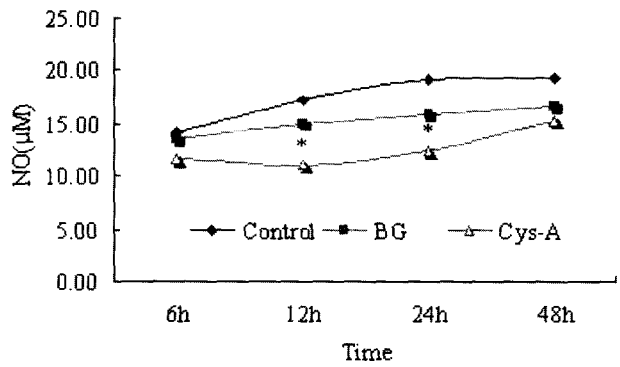

Fig. 1. The effect of bear's gall (BG) and cyclosporine on NO production from microglia. The addition of 1.2 ㎎/㎖ BG and 1㎍/㎖ cyclosporine to the rat microglia was observed for 48h and measurements were conducted at 48h using Griess reagent as indicated in company's instruction. *Value significantly different between BG and cyclosporine at each timepoints

2. EIA assay

To investigate the production of proinflammatory cytokine, IL-1β, at the level of protein, we set and designed the EIA polyclonal measurement for the detection of IL-1β released out to media from microglial cells. Four groups were designed for culture media, LPS single, LPS^+ bear's gall, and LPS^+cyclosporine and cultured for 48h (Fig. 5). Prior to that, we observed the bear's gall and cyclosporine single group in comparison with the normal control (Fig. 4). Results showed that the lowest IL-1β was detected at 12h in cyclosporine group while the tendency was gradually decreased as time went by not likely bear's gall. In Aβ42 group, IL-1β was weakly increased from 6h to 12h but was significantly increased at 24h and 48h comparing to the normal control (Fig. 4). When comparing each combination group with others, for example $Aβ42^+$bear's gall and $Aβ42^+$cyclosporine, IL-1β was decreased more in $Aβ42^+$Cyclosporine group than that in $Aβ42^+$bear's gall. However, this pattern was reversed at 48h (Fig. 6). Moreover, to investigate the data from the time courses in various concentrations for IL-1β, we set the time from 6h to 48h and the concentration from 0.8 ㎎/㎖ to 0.8 g/㎖ ranges. IL-1β was not significantly affected by 0.8 ㎎/㎖ bear's gall, but was significantly reduced in 1.2 ㎎/㎖ bear's gall. In contrary, higher concentration, 2.0 ㎎/㎖ and 0.8 g/㎖, did not show any better results as the two lower concentrations. In addition, the reduction of IL-1β in combination with LPS and Aβ42 at various bear's gall concentrations was significantly initiated from 1.2 ㎎/㎖ (data not shown).

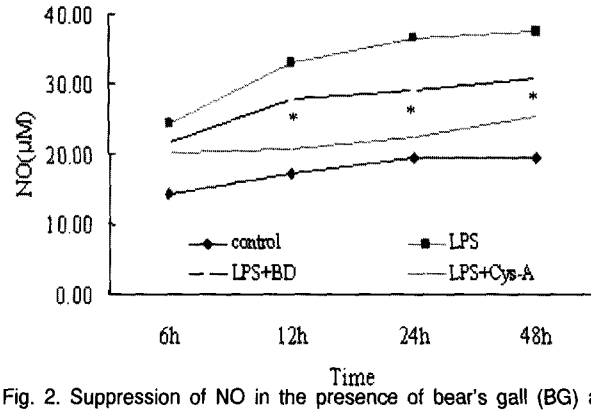

Fig. 2. Suppression of NO in the presence of bear's gall (BG) and cyclosporine in LPS pre-treated microglia. The addition of 1.2 ㎎/㎖ BG and 1㎍/㎖ cyclosporine to the rat microglia was observed for 48h and measurements were conducted at 48h using Griess reagent as indicated in company's instruction. *Value significantly different between LPS+BG and LPS+cyclosporine at each timepoints.

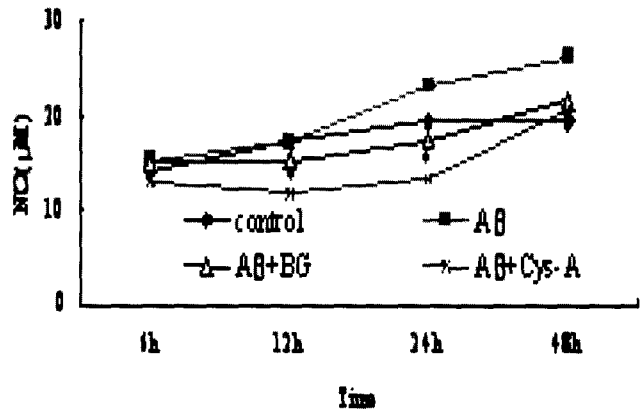

Fig. 3. Suppression of NO in the presence of bear's gall (BG) and cyclosporine in A42 pre-treated microglia. The addition of 1.2 ㎎/㎖ BG and 1㎍/㎖ cyclosporine to the rat microglia was observed for 48h and measurements were conducted at 48h using Griess reagent as indicated in company's instruction. *Value significantly different between Aβ42+BG and Aβ 42+Cyclosporine at each timepoints.

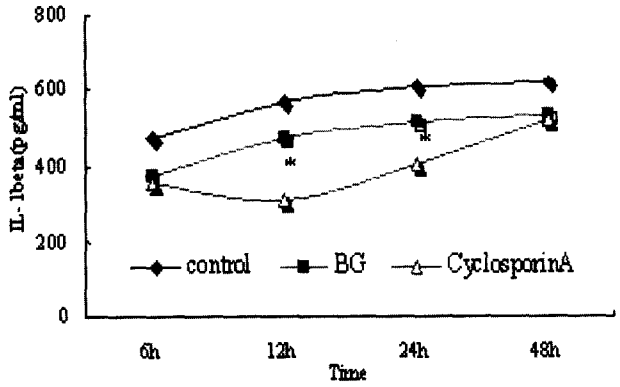

Fig. 4. Suppressive effect of bear's gall (BG) and cyclosporine in microglia. The addition of 1.2 ㎎/㎖ BG and 1㎍/㎖ cyclosporine to the rat microglia was observed for 48h and measurements were conducted at 48h using EIA detention kit as indicated in company's instruction. *Value significantly different between BG and cyclosporine at each timepoints.

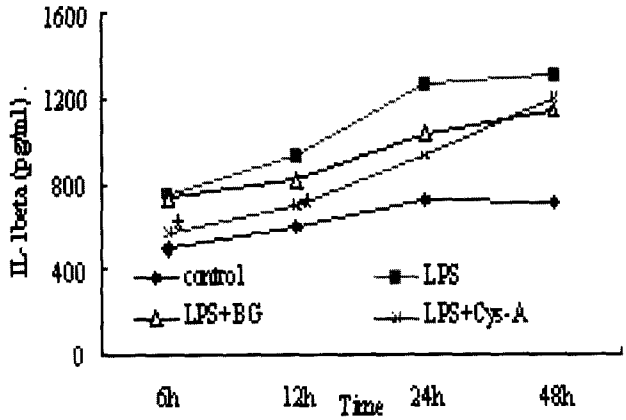

Fig. 5. Suppressive effect of bear's gall (BG) and cyclosporine in 10 ㎍ LPS-activated microglia. The addition of 1.2 ㎎/㎖ BG and 1㎍/㎖ cyclosporine to the rat microglia was observed for 48h and measurements were conducted at 48h using EIA detention kit as indicated in company's instruction. *Value significantly different between BG and cyclosporine in the presence of LPS at each timepoints.

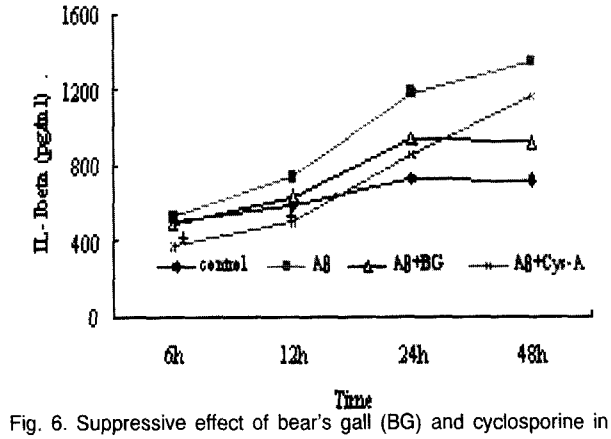

Fig. 6. Suppressive effect of bear's gall (BG) and cyclosporine in 5 μM fAβ42-activated microglia. The addition of 1.2 ㎎/㎖ BG and 1㎍/㎖ cyclosporine to the rat microglia was observed for 48h and the measurement was conducted at 48h using EIA detention kit as indicated in company's instruction. *Value significantly different between BG and cyclosporine in the presence of fAβ 42 at each timepoints.

3. T-PCR

For RT-PCT investigation, we uniformly set all the experimental conditions required for the process of RT or PCR and the final dose of bear's gall ranged from 0.8 ㎎/㎖ to 0.8 g/㎖. From this available range, we adopted 1.2 ㎎/㎖ because 1.2 ㎎/㎖ of bear's gall showed more stable data when the experiment was repeatedly conducted, and because we wanted to determine the most available dose for suppressing the release of proinflammatory cytokine from microglia. Cells were adjusted to a density of 1×10^5 in each experimental well by counting cells under the glass cell counter. After isolating and placing the cells on culture wells (6, 12, or 24-well plates), we stabilized them for at least 12 hours in the same condition as the primary culture. RT-PCR schedules were administered on the same day in order to reduce any bias resulting from the handling difference. 5 μM fAβ42 was prepared by incubating the peptides for at least 12 hours in 37℃ with gentle mix by tapping smoothly two or three times during incubation at low pH (<6.0). Additionally, 1g cyclosporine was used as a positive control. The results are shown in Fig. 7. β-actin was used for an indicator of the study validation.

Treatment of rat microglia with bear's gall, cyclosporine, and fAβ42 resulted in different gel bands according to their stimulating activities. As shown in Fig. 7, fAβ42 were strong stimulants for interleukin-1β (IL-1β), but bear's gall and cyclosporine in combination with fAβ42 suppressed IL-1β when comparing to normal control and fAβ42 single treatment for given chasing times (6h, 24h, and 48h). The strongest result was found in cyclosporine group in the beginning phase for 6h and 12h, but the trends were reversed between bear's gall and cyclosporine group at the end phase of the study (Fig. 7). Moreover, the continuity of efficacy was superior in bear's gall group to cyclosporine, which was corresponding to the EIA results as well as NO measurements.

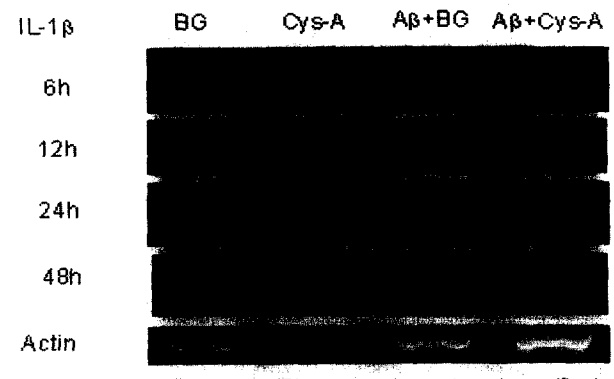

Fig. 7. Measurement of IL-1β mRNA expression and semi-quantification analysis of density at each sample group. The expression of IL-1β was measured by PCR amplification from SD rats as indicated in materials and methods. Lane 1: Bear's Gall (BG) (1.2 ㎎/㎖); Lane 2: cyclosporine (1 ㎍/㎖); Lane 3: fAβ42+BG; Lane 4: fAβ42+cyclosporine.

Dicussion

Microglia participate in an inflammatory response, signaling other glial and neuronal cells via cytokines by secreting a variety of immune-related substances such as complement, generate free radicals, as well as act as a clean-up crew in charge of clearing amyloid deposits. Reactive microglial products mediate activation of astrocytes as well as neuronal injury. Substances that lead to the activation of microglia (e.g. LPS) are correlated with neuronal toxicity[19], and differences in the density of microglia may explain why some part of the brain are more prone to inflammation or found to develop a higher density of senile plaques[9]. Microglial cytotoxicity has bolstered the concept that AD may be a form of chronic inflammation, and evidence that supports this hypothesis is the appearance of inflammatory markers in plaque-lesioned areas. Additionally, because microglia are the main inflammatory response cells of the brain and activated microglia are enriched around amyloid plaques, the microglia could make the areas around the plaques sites of chronic inflammation which may lead neurocytotoxicity[6,20]. When initiated, inflammatory cytokines produced activated microglia amplify and sustain inflammatory and immune responses[21], and may interact directly or indirectly with neurons[22]. Therefore, inflammatory responses of microglia may elicit deleterious effect on neurons.

A broad inflammatory repertoire, including NO, IL-1β, TNF-α levels even though IL-6 and macrophage colony stimulating factor (M-CSF) may be involved, is secreted by microglia and astroglia in the AD brain[23]. Of this inflammatory repertoire, IL-1β is a form of the two, IL-1α and -1β, whose biological activities are difficult to differentiate. Additionally, IL-1β, secreted predominantly by reactive microglia, occurs at elevated levels early in the development of a plaque and activates the production and processing of APP in the tissue, leading to a possible increase in Aβ production[24,25]. In this way, IL-1β contributes to the formation of new sources of amyloid in a kind of positive feedback that can accelerate formation of plaques and destruction of neurons. IL-1β activates astrocytes, promoting their secretion of IL-6, TNF, and S100-beta[26,27]. IL-1β has also shown to be directly toxic to neurons (in vitro) at high concentrations[26]. Evidence thus points to IL-1β as a major factor driving the disease[27]. On the other hand, NO, the product of a five-electron oxidation of amino acid L-arginine mediated by nitric oxide synthesis, is a major mammalian secretory product that initiates host defense, homeostatic and developmental functions by either direct effect of intracellular signaling and is a key molecule that stimulates host defenses in the immune system, maintains blood pressure in the cardiovascular system and modulates neural transmission in the brain. As a direct effector, NO is thought to activate regulatory proteins, kinases and proteases that are directed by reactive oxygen intermediates. In particular, NO acts as a neurotransmitter in the central and peripheral nervous systems and, therefore, is critical in the pathogenesis of stroke and other neurodegenerative disorders[28]. As to neurodegerative disorder, Alzheimer's disease is linked to NO, and the Aβ-associated free radical oxidative stress plays a pathological role in neurotoxicity[29].

In our experiments, we found that IL-1β and NO was suppressed by bear's gall as well as cyclosporine, a positive control in cultures pre-treated with fAβ42 and LPS. Eventually, cyclosporine was the strongest suppressor of NO and IL-1β, but bear's gall also showed the close results and even lasted its effect longer than cyclosporine. Namely, when activated with fAβ42 and LPS before adding bear's gall, NO and IL-1β were significantly inhibited comparing with the normal control. Moreover, bear's gall revealed the most interesting result, which showed reversed values comparing with cyclosporine when the time went by (Fig. 3, 6, 7). This may imply that bear's gall gradually played a role in suppressing NO and IL-1 β and its role lasted for longer time (48h). In the study with LPS, we found a similar outcome that suppressed microglia by co-culture with bear's gall, and its efficacy exceeded cyclosporine at 48h in both NO and IL-1β measurement except for NO with LPS. (Fig. 2) As NO and IL-1β are generally considered as proinflammatory mediators and oxidative stressors in cells, their expression in nuclear or the production out to extracellularly, have something great to do with cell damage and neurodegenerative disease which are the major cause of the symptom. Therefore, the results found in our experiments seem to give a certain cue in AD therapy in that bear's gall plays an important role in suppressing those AD causatives provoking cell damage or cell death when stimulated by massive A peptides through microglia. In turns, results from the combination with bear's gall in pre-treated with fAβ42 are thought to correlate with the signals of transcription factor NF-κB, which is activated by oxidative stressors including NO and IL-1β[30].

In conclusion, a significant anti-inflammatory effect of bear's gall in vitro suggests that it may regulate the expression or the production of inflammatory mediators, and this would be a cue to protect neurons from the cell death or damages in the chronic stage of AD caused by activated microglia in the presence of massive Aβ peptides. Therefore, if AD is caused by a chronic inflammation damaging neurons, bear's gall

would be a future candidate for the AD treatment programs by suppressing the pro-inflammatory mediators.

Acknowledgement

This study was supported by grants of Sangji University in 2002.

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