Potential Role of Anti-inflammation by Red Ginseng in Rat Microglia

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The most common feature of neurodegenerative disease (i.e. Alzheimer's disease, AD) is the increased number of activated microglial cells nearby the pathogenic area of the brain, such as amyloid plaque in AD. An abnormality of protein regulation and an imbalance of clearance against β-amyloid (Aβ) produced amyloid precursor protein (APP) can turn microglia into the activated feature out of the ramified resting phase. We examined the possibility that ginsenoside Rb1 could attenuate the microglial activation induced by massive Aβ that has known to induce a chronic inflammation, which is a major cause of AD by damaging neuronal cells (i.e. apoptosis or necrosis). Aggregated Aβ42 (5 μM) peptide was used with lipopolysaccharide (LPS) (10 ㎍) for a comparative control up to 48hours. We found that Rb1 reduced the production of nitric oxide as well as proinflammatory cytokines, such as IL-1β and TNF-α.

Key words : Alzheimer's disease, β-amyloid, ginsenoside Rb1, lipopolysaccharide, IL-1β, TNF-α

Introduction

In recent studies, inflammatory responses from microglia in AD have been a predictable road for research in AD[1]. When the brain is injured or affected by brain disease, the resident ramified microglia transform to "activated microglia" and produce proinflammatory cytokine such as IL-1β, TNF-α, nitric oxide that iNOS induces as a result of microglial activation. Those cytokines are often implicated as key mediators in the biological response to bacterial LPS, infection, and inflammatory stimuli. Inflammatory repertoires such as nitric oxide (NO), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) levels even though IL-6 and macrophage colony stimulating factor (M-CSF) may be involved, are secreted by microglia and astroglia in the AD brain[2]. Microglia or astrocytes, larger glial cells in the brain, have all been identified as source of cytokines affecting CNS-specific inflammation, but both in disease conditions and in culture systems, microglia, rather than astrocytes, appear to be the principal source of critical proinflammatory cytokines (IL-1β, TNF-α)[3,4]. In addition to such cytokines, nitric oxide is one of AD-related factors in development of pathogenesis. In other words, the inducible nitric oxide synthase (iNOS), which produces NO at enhanced rates, is expressed upon stimulation in many tissues, including the CNS[5]. Particularly, in chronic disease, inflammatory reactions are long lasting and accompanied by activation of microglia, and those activated microglia in culture have shown to produce several potential cytotoxic molecules, including superoxide anion, nitric oxide, and proinflammatory cytokines[6]. Those evidences that IL-1β, TNF-α and NO are all interconnectively related with AD, can give important opportunities in AD therapy in that significant reduction or suppression of expression of those proinflammatory factors may be a clue on drug development. In this research background, we wanted to investigate the potential effect of anti-inflammation by red ginseng. Therefore, we adopted these possibilities to the study theme and the study was designated to evaluate mRNA expression, the level of cytokine production, and the level of NO.

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Materials and methods

1. Primary cell culture

Microglial cells for primary culture were isolated from mixed glia prepared from newborn Sprague-Dawely rat (1-day-old, SD rat) as described in the lab protocol, which has been modified from previous reports[7]. We obtained microglia by confirming the cell quantity and quality under microscopy at various powers. The fully grown cells (>14days) were collected from culture flasks by shaking, centrifuged at 1450 rpm for 5 minutes, resuspended the pellet in 5% FBS-DMEM,

and were placed on the designed study plates (6, 12, 24, or 96 wells). Prior to placing cells on wells or slides, cell quantity was confirmed by counting with cell-counter averaged from 4 areas of the glass compartments, and density was adjusted to 1×10^5 cells/well. Cells were then stabilized for at least 2 hours before the start of experiment at 37℃ in a 5% CO_2 humidified incubator.

2. RT-PCR

Total RNA was extracted using the TRIZOL method as described previously with some modification[8]. 2 ㎍ of total cellular RNA were reverse transcribed for 20 ㎕ reaction using RT Premix, 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 placed 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 spin down by mini-centrifuge. cDNA synthesis reaction was performed at 4 2℃ 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 ㎕ of the following reaction mixture: PCR buffer (10mM Tris-HCl, 40mM KCl, 1.5mM $MgCl_2$, pH 9.0) 250 μM dNTP, 1unit Taq DNA polymerase, 2 ㎕ 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 TNF-α, 5′- ACT CCA GGC GGT GTC TGT GC-3′ and 5′-TGG CAA ATC GGC TGA CGG TG-3′ (411 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). Reaction products were separated by electrophoresis on a 2.0% agarose gel containing ethidium bromide (Sigma, U.S.A.) in buffer (40mM Tris-acetate and 1mM EDTA, pH 9.0), visualized in U.V. plate, photographed.

3. Cytokine measurement

EIA kits (TiterZyme® EIA, Assay Designs, U.S.A.) were used for the quantitative determination of level of the cytokines (IL-1β and TNF-α) 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.

Results and Discussion

A broad inflammatory repertoire, such as NO, IL-1β, and TNF-α, is secreted by mainly microglia in the AD brain[2,9] and the centers of this inflammatory response in AD are the senile plaques, which are extracellular deposits composed mainly of insoluble aggregates of Aβ protein surrounded by reactive glial cells[10,11]. Cytokines are key regulators of innate and adaptive immune responses. In infectious and autoimmune diseases of the central nervous system (CNS), tissue infiltrating immune cells, CNS-associated macrophages, microglia, and astrocytes have all been identified as sources of cytokines affecting CNS-specific inflammation. However, both in disease conditions and in culture systems, microglia, rather than astrocytes, appear to be the principal source of critical proinflammatory cytokines, IL-1β and TNF-α[3,12]. IL-1β and TNF-α are two major proinflammatory cytokines with pleiotropic and largely overlapping functions, produced by microglia and blood-derived macrophages during CNS inflammation. On the basis of in vitro evidence, microglia cells are also considered the major CNS sources of pleiotropic cytokines that stimulate humoral and cell-mediated immune responses. Importantly, IL-1, and TNF- are present at elevated levels in the AD brain[13]. As shown in Fig. 1. we found that the three inflammatory repertoire (NO, IL-1β, and TNF-α) was attenuated by red ginseng in activated microglia by Aβ42 peptide dissolved in 5% FBS culture mediator. In particular, similar patterns for time courses between $A\beta^+$red ginseng and $A\beta^+$LPS were found from the initiation time phase up to 48h, and the intervals between the two were maximized at 48h. Interestingly, non-aggregated Aβ42 could not activate microglia as much as LPS (data not shown). However, when time went further, it seemed that Aβ42 peptide naturally aggregated to some extents under the incubation condition. This resulted in the increase of NO, IL-1β, and TNF-α at 48h (Fig. 1, 2, 3). In addition, the mRNA expressions of IL-1β, and TNF-α were also corresponding to the results of EIA detection (Fig. 2, 3).

In conclusion, we have found that microglia cells are

activated by LPS and Aβ for the indicated time courses. However, red ginseng combined with Aβ showed a significant inhibition effect on IL-1β, TNF-α, and NO in all test experiments such as NO detection, RT-PCR, and EIA assay. The fact that red ginseng downregulates the release of such proinflammatory repertoire suggest that it might be possible to inhibit the repertoires and protect the neuronal damage. Further research will be required to elucidate the detailed intra- or extra-cellular actions of red ginseng and to determine whether red ginseng might act as a signal mediator in neurodegenerative processes such as AD.

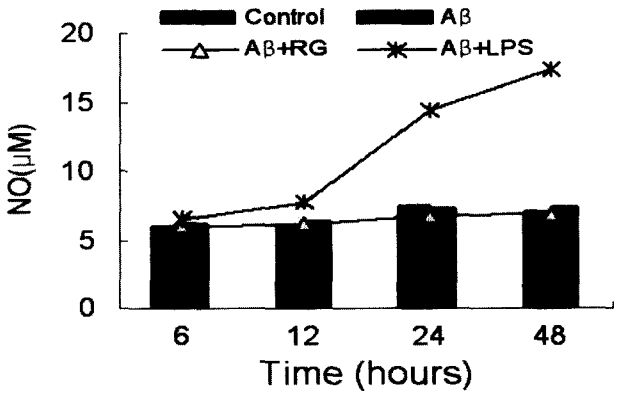

Fig. 1. Suppression of nitric oxide in microglia when activating with A β in the presence or the absence of red ginseng. Nitric oxide detected by griess reagent system. A: β-amyloid (Aβ42); RG: red ginseng; LPS: lipopolysaccharide, 5 μM, 100 μM, and 10g/ml, respectively. Results are means ± SD.

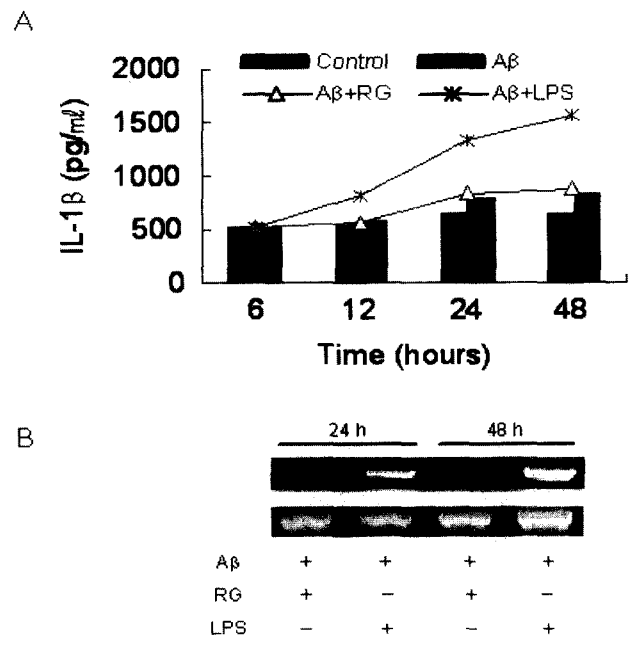

Fig. 2. Suppression of IL1-β in microglia when activating with Aβ in the presence or the absence of red ginseng. (A) IL-1β by EIA detection kits; (B) IL-1β mRNA expression by PCR amplification. Aβ: β amyloid (Aβ42); RG: red ginseng; LPS: lipopolysaccharide, 5 μM, 100 μM, and 10 ㎍/ml, respectively. Results are means ± SD.

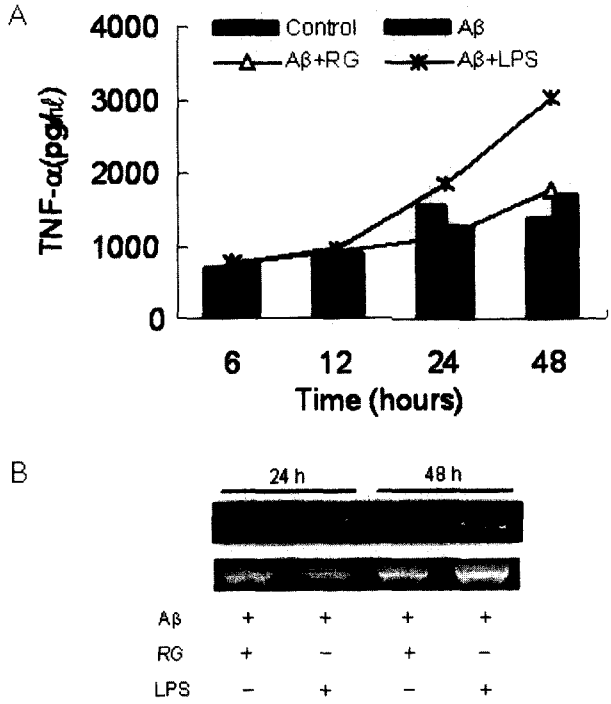

Fig. 3. Suppression of TNF-α in microglia when activating with Aβ in the presence or the absence of red ginseng. (A) TNF-α by EIA detection kits; (B) TNF-α mRNA expression by PCR amplification. Aβ: β-amyloid (A β42); RG: red ginseng; LPS: lipopolysaccharide, 5 μM, 100 μM, and 10㎍/ml, respectively. Results are means ± SD.

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