Prevention of Inflammation-Mediated Neurotoxicity by Rg3 and Its Role in Microglial Activation

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Considering the importance of inflammation and apoptosis in neurodegenerative conditions, the potential suppressive effects of the Rg3, a by-product obtained during the steaming of red ginseng, may indicate that Rg3 could provide a beneficial therapeutic approach to treating or preventing neurodegenerative disease. We investigated the effect of Rg3 on Aβ42-mediated microglial activation and inflammation-mediated neurotoxicity in murine BV-2 microglial and Neuro-2a neuroblastoma cells, respectively. Rg3 effectively reduced inflammatory cytokine expression in Aβ42-treated BV-2, and inhibited the binding of NF-κB p65 to its DNA consensus sequences, and significantly reduced the expression of TNF-α in activated microglia. Pretreatment with Rg3 increased the survival rate of Neuro-2a exposed to TNF-α. These observations suggest that Rg3 reduced neurotoxicity by inhibiting chronic inflammation through the suppression of activated microglia. In addition, the expression of pro-inflammatory cytokines in BV-2 stimulated by Aβ42 was decreased but not eliminated by Rg3 when binding to the macrophage scavenger receptor type A (MSRA) was blocked with fucoidan. This implies that the inflammatory response may not be exclusively triggered via MSRA. Moreover, Rg3 decreased the expression of MSRA in BV-2 transfected with siRNA targeting MSRA mRNA, and this increased MSRA expression may play a role in the phagocytosis of Aβ42 peptides. Our results indicate that inhibition of the inflammatory repertoire of microglia, neuroprotection, and increased MSRA expression induced by Rg3 may at least partly explain its therapeutic effects in chronic neurodegenerative diseases.

Key words microglia; Rg3; beta amyloid; chronic inflammation; macrophage scavenger receptor type A

Inflammatory processes occurring in the CNS are closely related to pathways leading to neuronal cell death in many types of neurodegenerative conditions such as Alzheimer’s disease (AD), Parkinson’s disease, prion disease and multiple sclerosis. In particular, chronic inflammation in the brain is harmful because of the possible vulnerability of neurons to the inherent damage caused by inflammatory processes.1) The inflammatory response in the brain is mediated by the antigen-presenting brain immune cells, activated microglia, that normally respond to neuronal damage and remove damaged cells by phagocytosis.2,3) Although the microglial activation is regarded as a hallmark in the field of brain pathology and their activation is critical for host defense, chronic activation of microglia may cause neuronal damage through the release of potentially cytotoxic pro-inflammatory cytokines and reactive oxygen intermediates.4,5) In AD, the imbalance between beta amyloid (Aβ) production and removal leads to the formation of fibrillar Aβ deposits and microglial activation, followed by the induction of a neuroinflammatory response, gliosis and the formation of dystrophic neuritis.6,5) Hence, determining the repressive effects of various drugs on the potential mechanisms and signaling pathways involved in the activation of microglial cells during CNS injury is critical to developing therapeutic approaches for neurodegenerative diseases. In previous studies, we have reported that ginsenosides from red ginseng have potential suppressive and anti-inflammatory effects on BV-2 cell.8) A follow-up study revealed that Rg3, a panaxadiol ginsenoside, prevents inflammation-induced damage from occurring in neuronal cells via phosphorylation of the cell survival protein Akt.9) In this study, we hypothesized that Rg3 suppresses chronic microglial responses, and thus protects neurons from chronic inflammation-mediated tissue damage induced by the aberrant production of Aβ42 in AD.10) We also investigated the Rg3-mediated reduction in the release of inflammatory cytokines in response to the direct binding of Aβ42 peptides to the macrophage scavenger receptor type A (MSRA) of microglial cells. In the present study, we investigated the expression profiles of inflammatory cytokines in BV-2 cells and determined whether Rg3 has a therapeutic role in the suppression of chronic activation of microglia and in protection from neuronal cell damage induced by cytotoxic cytokines, both of which are involved in neurodegenerative pathogenesis.

MATERIALS AND METHODS

Materials A ginsenoside, Rg3, derived from Panax ginseng at a purity of at least 98% (HPLC-MS), was kindly provided by the Ambo Research Center (Seoul, Korea) (Fig. 1).11) A concentrated stock solution (10 mg/ml) of Rg3 was prepared by dissolving in 10% dimethylsulfoxide (DMSO, Sigma, MO, U.S.A.) and was kept at 4 °C until use. The stock solution was further diluted in culture media at a 1:10 ratio

Fig. 1. Chemical Structure of Ginsenosides Rg3

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for cell treatments. We found that the final concentration of DMSO in the culture media (<0.25%) had no effect on microglial activation (data not shown). Lyophilized AB42 (Calbiochem, CA, U.S.A.) was diluted in distilled water to 5 mg/ml and stored at −20 °C until further use.

**Cell Culture** The BV-2 microglial cell line was grown in 10% FBS α-MEM (Cellgro, VA, U.S.A.) for 3 d, followed by a change of the media to 5% FBS α-MEM on the fourth day. All studies were performed using 2% FBS α-MEM media, following an overnight incubation period prior to use. For microglial activation, 5 μg/ml AB42 (Calbiochem) was uniformly added to the cell culture. Neuro-2a cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, GibcoBRL, MD, U.S.A.) supplemented with 5% heat-inactivated fetal bovine serum (GibcoBRL) at 37 °C, 5% CO2 and 95% air in a humidified cell incubator.

**Small Interfering RNA and Western Blot Analysis** The siRNA strategy was employed to silence the mouse MSRA in BV2 cells. MSRA siRNAs were generated using Donze’s procedure12) and the T7 RibomAX Express RNAi system (Promega, WI, U.S.A.). Briefly, the siRNA sequences for the mouse MSRAs and the primers were chosen using a web-based tool (siRNA Target Designer, Promega). The primers were as follows: MSRA sense, 5'-AAGAAATGCCTGGATCTCCTCATAGTGTACGTTATAGGATCC-3', and MSRA antisense 5'-AAGGAGAGAATCCAAAAGCATTCTATAGTGTCGTATAGGATCC-3'. The target sequences for the MSRA gene are underlined, and the remaining 3'-end regions of each primer correspond to the T7 promoter sequences. The T7 si primer, 5'-GGATCTCAATTACGACTCATATAG-3', was synthesized, and the oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase was carried out using the T7 RibomAX Express RNAi system (Promega). The sense and antisense 21-nucleotide RNAs, generated in separate reactions, were annealed by mixing both transcription reactions, incubating at 70 °C for 10 min and then at room temperature for 20 min to obtain the small interfering double-stranded RNA. Next, the mixture was purified by isopropanol precipitation, washed in 70% ethanol, dried and resuspended in an appropriate amount of nuclease-free water. The concentration of siRNAs was optimized to 2.5 μg in each transfection for BV2 cells using the CodeBreaker™ siRNA Transfection Reagent (Promega). Following a 48 h transfection period, western blots were performed by washing cells with PBS and subsequently lysing them in RIPA buffer containing protease- and phosphatase-inhibitors. In addition, 30 μg of each protein lysate were boiled for 5 min and then electrophoresed on a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF membrane, which was then blocked with TBS-T containing 5% skim milk. The membrane was incubated with an anti-MSRA polyclonal antibody (Santa Cruz, CA, U.S.A.). After washing with TBS-T, the membrane was incubated with a secondary antibody, rabbit anti-goat IgG conjugated with horseradish peroxidase (Zymed, CA, U.S.A.), for 2 h at room temperature. The membrane was then developed with ECL solution (Amersham Bioscience, NJ, U.S.A.).

**Cytokine Protein Array (Mouse Inflammation Antibody Array)** Multiple pro-inflammatory cytokines were simultaneously detected in cell lysates using a commercially available enzyme-linked immunosorbent assay (ELISA)-based cytokine protein array (Ray Bio cytokine array; Ray-Biotech, GA, U.S.A.) containing 32 pairs of captured monoclonal antibodies spotted onto a nitrocellulose membrane according to the manufacturer’s instructions. Briefly, cytokine array membranes were blocked in 2 ml of 1× blocking buffer for 30 min and then incubated with 1 ml of each sample at room temperature for 1 to 2 h. Sample solutions were then decanted and the membranes were washed three times with 2 ml of 1× wash buffer I, followed by two washes with 2 ml of 1× wash buffer II at room temperature with shaking. Membranes were then incubated in 1 : 250-diluted biotin-conjugated primary antibodies at room temperature for 1.5 h and washed as described above before incubation in 1 : 1000-diluted horseradish peroxidase-conjugated streptavidin. After incubation in horseradish peroxidase-conjugated streptavidin for 1 h, membranes were washed thoroughly and exposed to a peroxidase substrate (detection buffers C and D; Ray-Biotech, Inc.) for 5 min in the dark before imaging. Membranes were exposed to X-ray film (Kodak X-OMAT AR film) within 30 min of exposure to the substrate. The positive control signals on each membrane were used to normalize the cytokine signal intensities. Their intensities were determined by densitometric analysis using Gelquant software (MiniBIS Pro, Jerusalem, Israel). For each spot, the net optical density was determined by subtracting the background optical density level from the total raw optical density level.

**Cytotoxicity, ELISA and RT-PCR Assay** The cytotoxicity of Neuro-2a cells in response to TNF-α stimulation was investigated following a 24 h culture period using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). The system uses WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt], which produces a water-soluble colored formazan upon bioreduction in the presence of the electron carrier, 1-methoxy PMS.13) The reaction plates were measured at 450 nm and the data from triplicate cultures were expressed as percent viability. The TNF-α ELISA kit (Assay Designs, MI, U.S.A.) was used in the detection of secreted cytokine levels in the cell culture supernatants after 24 h incubation. The reactions were read at 450 nm using a microplate reader (SupraMax340, Molecular Devices, CA, U.S.A.). For RT-PCR (reverse transcription polymerase chain reaction), total RNAs were obtained from BV-2 cells using Trizol (Invitrogen, CA, U.S.A.). In brief, the cells were lysed using 1 ml Trizol reagent, followed by adding 200 μl of chloroform after a 1-min incubation at room temperature. The mixture was then centrifuged at 13500 rpm for 10 min and the aqueous layer was transferred to another tube. The RNA was then precipitated by centrifugation with isopropanol (500 μl) at 13500 rpm for 5 min. Next, the RNA pellets were washed in cold Et-OH (700 μl) (70% in DEPC water) and air-dried. The total reaction volume was 20 μl and the amplified cDNA was separated on a 1.2% agarose gel stained with ethidium bromide (Sigma). The PCR amplification was performed using the primers listed in Table 1 (Bioneer, Deajeon, Korea). The band intensities of the amplified DNA were compared using a gel documentation system (MiniBIS Pro, Jerusalem, Israel).

**Nuclear Factor Binding Assays** Analysis of the specific binding of p65 to DNA oligonucleotides containing its consensus binding site was performed in nuclear extracts using
Inhibited by the presence of Rg3, compared to the group.

Of the 32 inflammatory cytokines present on the array, IL-cytokine spectrum of each group is shown in Figs. 2A and B. Using a cytokine antibody array. The relative variability in the levels of 32 inflammatory cytokines in BV-2 cell lysates BV-2 cells in the presence or absence of Rg3, we examined differences in the specific expression of inflammatory cytokines. The cytokine array image of three antibody-coated membranes represents one of two independent experiments that found similar patterns of expression. The black circles represent the cytokine spots is normalized to the positive control in each sample set.

To determine differences in the specific expression of inflammatory cytokines, we used a cytokine antibody array. The relative variability in the levels of 32 inflammatory cytokines in BV-2 cell lysates BV-2 cells in the presence or absence of Rg3, we examined differences in the specific expression of inflammatory cytokines. The cytokine array image of three antibody-coated membranes represents one of two independent experiments that found similar patterns of expression. The black circles represent.

RESULTS

Inflammatory Cytokine Profile and NF-κB-Binding Assay in BV-2 Cells To determine differences in the spectra of inflammatory cytokines expressed by Aβ42-treated BV-2 cells in the presence or absence of Rg3, we examined the levels of 32 inflammatory cytokines in BV-2 cell lysates using a cytokine antibody array. The relative variability in the cytokine spectrum of each group is shown in Figs. 2A and B. Of the 32 inflammatory cytokines present on the array, IL-1α, IL-6, MCP-1 and MIP-1γ levels were found to be most inhibited by the presence of Rg3, compared to the group treated with Aβ42 alone. It is interesting that Rg3 effectively attenuated the Aβ42-stimulated inflammatory responses of microglia, suggesting that Rg3 may be involved in the process of inflammation. Coincidentally, we found that Rg3 effectively suppressed the binding of the NF-κB p65 subunit to its DNA consensus sequence in nuclear extracts after 24 h of co-treatment with Aβ42 peptides, relative to the binding of the NF-κB p65 subunit after 1 h of co-treatment, which remained unchanged (Fig. 3). This result implies that Rg3 might be associated with intracellular signaling via, for example, NF-κB pathways, but not with membranous receptors, such as MSRA.

Protection of TNF-α Mediated Cell Damage in Neuro-2a Cells To verify that Rg3 prevents the production of TNF-α in microglia, and increases neuronal cell survival in the ELISA-based TransAM NF-κB p65 transcription factor assay kit (Active Motif, CA, U.S.A.) according to the manufacturer’s instructions. Briefly, 5 μg of BV2 nuclear extract were added to 96-well plates coated with an oligonucleotide containing the nuclear factor consensus site (5′-GGGACTTTCC-3′). The binding of NF-κB to DNA was visualized using an anti-p65 antibody, which specifically recognizes activated NF-κB. Antibody binding was measured using a spectrophotometer within 5 min at 450 nm with a reference wavelength of 655 nm. The activation specificity of the nuclear factor was determined by competition experiments using wild-type and mutant consensus oligonucleotides provided with the Trans-AM transcription factor assay kit (Active Motif, CA, U.S.A.) according to the manufacturer’s instructions.

Table 1. Primer Sequences for the PCR Analysis

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Primer</th>
<th>Amino acid sequence</th>
<th>Ann. temp °C</th>
<th>Cycles</th>
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<td>mTNF-α</td>
<td>Sense</td>
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<tr>
<td>mIL-1β</td>
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<tr>
<td>miNOS</td>
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<tr>
<td>mβ-actin</td>
<td>Sense</td>
<td>5′-TGGACGAGCTGCTACAGC-3′</td>
<td>58</td>
<td>30</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of Rg3 on Aβ42-Induced Cytokine Profiles of a Murine Microglial Cell Line

BV2 cells were exposed to Aβ42, Rg3, or both for 24 h. Untreated cell cultures were used as a control and culture supernatants were subjected to a cytokine antibody array. (A) In the presence of Rg3, compared to the group treated with Aβ42 alone. It is interesting that Rg3 effectively attenuated the Aβ42-stimulated inflammatory responses of microglia, suggesting that Rg3 may be involved in the process of inflammation. Coincidentally, we found that Rg3 effectively suppressed the binding of the NF-κB p65 subunit to its DNA consensus sequence in nuclear extracts after 24 h of co-treatment with Aβ42 peptides, relative to the binding of the NF-κB p65 subunit after 1 h of co-treatment, which remained unchanged (Fig. 3). This result implies that Rg3 might be associated with intracellular signaling via, for example, NF-κB pathways, but not with membranous receptors, such as MSRA.

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![Fig. 2](image)

![Fig. 3](image)
the presence of TNF-α, BV-2 and Neuro-2a cell lines were examined individually (Figs. 4A, B). The exposure of BV-2 cells to Aβ42 was found to increase the concentration of TNF-α in the culture media. However, this up-regulation was significantly attenuated at 24 h after treatment with Rg3 (10 μg/ml) (Fig. 4A). Next, we examined whether Rg3 increases the cell survival rate in the presence of TNF-α, which presumably induces apoptotic cell death. Figure 4B revealed that Rg3 effectively increased cell survival even at the lower concentration of Rg3 (5 μg/ml), suggesting that Rg3 could effectively protect neuronal cells from the neurotoxic cytokine, TNF-α.

**Inhibition of the Microglial Pro-inflammatory Repertoire**

The binding of Aβ42 peptides labeled with a fluorescent cyanine probe, Cy3, to BV-2 cells treated with the MSRA ligand, fucoidan, was investigated (Fig. 5A). The results suggest that fucoidan completely blocks MSRAs on microglia. In addition, cytokine and iNOS suppression by Rg3, which are all directly involved in inflammatory pathogenesis, were investigated in cells pre-treated with fucoidan to block MSRA binding by Aβ42. The results revealed that Rg3 effectively suppressed the Aβ42-induced expression of cytokines and iNOS (Fig. 5B).

**Inhibition of Mouse MSRA Synthesis Using siRNA**

In order to determine whether Rg3 contributes to the expression of MSRA in BV-2 cells, we constructed a small interfering RNA (siRNA) against MSRA (Figs. 6A, B). Western blot analysis revealed that a significant reduction (>50%) of the MSRA protein was achieved in microglia transfected with the siRNA construct and treated with Aβ42 alone, whereas incubation of the siRNA transfected cells with both Rg3 and Aβ42 was found to restore MSRA expression, presumably by overwhelming the siRNA degradation machinery with...
highly induced levels of MSRA mRNA (Fig. 6).

DISCUSSION

In neurodegenerative diseases, the activation of microglia aberrantly responding to Aβ42 peptides is the main source of secreted pro-inflammatory cytokines, TNF-α and IL-1β in the brain.13,16 Moreover, IL-1β, TNF-α, and Aβ42 may stimulate microglia to produce chemokines such as MCP-1 and MIP-1α.16,17 Therefore, the inhibition of Aβ42-induced cytokine and chemokine production by microglia can provide a novel and rational therapeutic approach to the inflammation-induced progression of AD. More importantly, AD-activated microglia, which take part in inflammatory responses, may also trigger signals in other glial and neuronal cells.18 In addition, uncontrolled chronic neuroinflammation is now known to play a key role in the progression of damage in a number of neurodegenerative diseases. Therefore, the overproduction of pro-inflammatory cytokines and iNOS may provide a pathophysiological progression mechanism for the development of anti-neurodegenerative drugs.19,20 The progressive interaction between activated microglia and inflammation-mediated neurotoxicity can be a major contributor to neurotoxin-induced neuronal cell death, which in turn leads to disease states, such as AD.21 Previously, we have reported that Rg3 enhances the phagocytosis of microglia,22 and prevents neuronal apoptosis.23 Based on the results of the present study, we expect that since Rg3 suppresses the Aβ42-induced mass production of the pro-inflammatory repertoire of microglial cells via down-regulation of NF-κB DNA-binding, then Rg3 should protect against activated microglial-induced neuronal cell death in neurodegenerative diseases. Importantly, TNF-α is known to promote cell death during neuroinflammatory and neurodegenerative disease, and there is abundant evidence suggesting that TNF-α has an overall neurotoxic effect.23 In our study, we observed that Rg3 may rescue TNF-α-induced neuronal cell death. Further evaluations will be needed to elucidate the actual role played by Rg3 in neuronal cell survival, but one possible hypothesis is that Rg3 effectively suppresses activated microglia, and thus releases the reduction of inflammatory cytokines, including neurotoxic TNF-α. Effective suppression by Rg3 of the binding of the NF-κB p65 subunit to its DNA consensus sequence following stimulation of BV-2 cells with Aβ42 peptides supports this potential preventive effect on inflammation-mediated neurotoxicity. Interestingly, we also found that Rg3 can synergistically attenuate Aβ42-mediated microglial activation when the cells have been pre-treated with fucoidan, a MSRA ligand.24 These results demonstrating that Rg3 suppresses the inflammatory repertoire of microglial cells, may offer new information towards understanding the mechanistic involvement of Rg3 with MSRA. Moreover, the inhibition of iNOS by Rg3 in the presence of fucoidan strongly suggests that Rg3 can play an important role in the selective inhibition of iNOS expression in microglia.25 Aβ42-induced mRNA expression of MSRA in BV-2 cells was significantly increased by Rg3 despite the expression of MSRA siRNA in these cells. This indicates that Rg3 can stimulate MSRA expression independently of cell surface interactions. As our previous study reported, the increased MSRA levels on microglia may enhance the removal of Aβ42 peptides from the brain.23 In conclusion, it is noteworthy that we consider the role of chronic microglial activation and inflammation-mediated neurotoxicity as key characteristics in the pathogenesis of neurodegenerative diseases, and thus the development of candidate drugs that can potentially protect neurons against activated microglia and neurotoxin-induced neuronal cell death is of critical importance. The findings from our research indicate that Rg3 can effectively attenuate Aβ42-activated microglia-induced neurotoxicity and may potentially protect against microglia-mediated neurodegenerative diseases, although further in-depth in vivo research is necessary to confirm these findings.

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