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Potential Effects of Microglial Activation Induced by Ginsenoside Rg3 in Rat Primary Culture: Enhancement of Type A Macrophage Scavenger Receptor Expression

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Brain microglia are phagocytic cells that are the major inflammatory response cells of the central nervous system and widely held to play important pathophysiologic roles in Alzheimer's disease (AD) in both potentially neurotoxic responses and potentially beneficial phagocytic responses. In the study, we examined whether ginsonoside Rg3, a by-product of red ginseng, enhances the microglial phagocytosis of Aβ. We found that Rg3 promoted Aβ uptake, internalization, and digestion. Increased maximal Aβ uptake was observed at 4 and 8 h after Rg3 pretreatment (25 μg/mL), and the internalized Aβ was almost completely digested from cells within 36 h when pretreated with Rg3 comparing with single non-Rg3-treated groups. The expression of MSRA (type A MSR) was also up-regulated by Rg3 treatment in a dose- and time-dependent manner which was coincidently identified in western blots for MSRA proteins in cytosol. These results indicate that microglial phagocytosis of Aβ may be enhanced by Rg3 and the effect of Rg3 on promoting clearance of Aβ may be related to the MSRA-associated action of Rg3. Thus, stimulation of the MSRA might contribute to the therapeutic potentials of Rg3 in microglial phagocytosis and digestion in the treatment of AD.

Key words: Microglia, Macrophage scavenger receptor, Aβ42, Ginsenoside Rg3, Alzheimer's disease

INTRODUCTION

Microglial cells, the main immune effector cells of the brain and the resident antigen-present cells (APC) in the CNS, usually exist in a ramified resting status, but in nearly all brain pathologies, microglial cells are rapidly and easily activated, changes their morphologies to an amoeboid shape, increases phagocytic activities and stimulates the production of functional mediators such as cytokines and growth factors (Giulian *et al.*, 1993; Kreuzberg *et al.*, 1996; Hailer *et al.*, 2001). Particularly, activated microglia participate in an inflammatory response, signaling other glial and neuronal cells *via* various immune-related substances, generate free radicals, and also act as a clean up crew in charge of clearing amyloid deposits in Alzheimer's disease (AD) (Edelstein-keshet and Spiros, 2002). Importantly, the presence of a chronic inflammatory response has been characterized in the brains of AD patients and hypothesized that this inflammation is a response to plaques from the aggregation of beta amyloid (Aβ) peptides (Dickson, 1997). In previous studies, it has been demonstrated that microglia activated by Aβ migrated and surrounded the region of compact Aβ deposits and Aβ stimulated phagocytosis of microglia (Kopec and Carroll, 1998; Chung *et al.*, 1999). There is no doubt that Aβ plaques and Aβ itself induce the activation of microglia and such activation contributes to the inflammation process in AD (Kitamura *et al.*, 1999). However, the clearance of brain Aβ (e.g. Aβ42) may also be significant in studying the biochemical pathogenesis of AD because AD involves the abnormal accumulation of Aβ. In many studies, it has been demonstrated that activated microglia are capable of releasing cytotoxic agents such as proteolytic enzymes, cytokines, complements proteins, reactive oxygen intermediates, and nitric oxide when stimulated by Aβ peptides (Giulian *et al.*, 1995), which is mainly harmful, but they could also play a protective role by digesting amyloid fibrils as described above. Therefore the prompt clearance

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of Aβ peptides as it happens in normal brain could be the key point in AD prevention. In the AD brain, microglial clearance of Aβ is not at sufficiently rapid rate to prevent overall accumulation (Rogers *et al.*, 2002). Cell surface receptors that have known to be associated with the clearance Aβ include the formyl peptide receptor (FPR) (Lorton *et al.*, 2000), macrophage scavenger receptor (MSR) and the receptor for advanced glycation end product (RAGE), all of which have Aβ as a ligand and are expressed by microglia (Yan *et al.*, 1997). Among those receptors, MSR is an important factor in Aβ responses by microglia (Rogers *et al.*, 2002) as well as in the uptake of fibrillar Aβ (Paresce *et al.*, 1996). Although there are two classes of MSR, class A (MSRA) and class B (MSRB), that could mediate uptake of Aβ, it seems likely that MSRA is the MSR that plays a significant role in Aβ clearance in the brain (Paresce *et al.*, 1996) because only MSRA has been shown to be upregulated and only on microglia in AD pathological states (Husemann *et al.*, 2002). In the study, we scrutinized the potential effect of the purified form of ginsenoside Rg3, a panaxadiol, on the MSRA expression on microglial cell surface and the enhancement of Aβ uptake in the presence of Rg3. In previous studies, ginsenoside, a ginseng glycoside, showed potential effects on the CNS (Tsang *et al.*, 1985), antineoplastic and immunomodulatory effects (Wagner *et al.*, 1994), and stimulation of phagocytosis (Shibata *et al.*, 2001). Little is known about the pharmacological action of ginseng, but the suggested fundamental mode of action is that it has an "adaptogenic" effect (Wagner *et al.*, 1994), which produces a non-specific increase in the body's own defenses against exogenous stress factors and noxious chemicals and that it promotes an overall improvement in physical and mental performance (Phillipson *et al.*, 1984). Among the various applications of ginseng, immunomodulatory activity is considered to be the meaningful response of ginsenoside's adaptogenic effect in the defense mechanism, which may stimulate phagocytosis and the production of interferon, and enhance the activity of natural killer cells (Singh *et al.*, 1984). In the present study, we studied the potent ability of Rg3 whether it stimulates the MSRA expression and increases the phagocytosis of microglia along with an enhanced digestion of Aβ in rat primary culture.

MATERIALS AND METHODS

Cell culture

Microglia were isolated from mixed glial cultures which had been prepared from newborn Sprague-Dawley rat brains, as was previously described (Chung *et al.*, 1999). The mixed glial cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL of streptomycin (Life Technologies, U.S.A.) at 37°C for 10-14 days in a 5% CO_2 humidified air atmosphere, after which the microglia were harvested. The microglia were harvested by 15-20 minutes of orbital shaking at 200 rpm, and were then centrifuged for 3 minutes at 1350 rpm, and plated on six-well tissue culture plates at 1.5×10^5 cells/well, or onto four-well chamber slides at a density of 1×10^4 cells/well.

Protein preparation and microscopic observation

The Aβ1-42 (Aβ42) peptides were purchased from Bachem, UK. Fluorescently-labeled Aβ42 was prepared via conjugation with Cy3, a carbocyanine dye (Amersham Biosciences, UK), as was previously described (Paresce *et al.*, 1997). Fluorescent acetylated LDL complexes (DiI-Ac-LDL) were purchased from Molecular Probes (U.S.A.), and fucoidan, a polysaccharide ligand for MSRs, was purchased from Calbiochem (Germany). In order to visualize the microglial binding and uptake of Aβ42, the cells were grown in four-well chamber slides for 24 h prior to the experiment. The cells were then refreshed with 1% FBS DMEM media, and treated with Rg3 (1-25 μg/mL) for 1, 4, or 8 h, and were then subsequently rinsed three times with 1 × phosphate-buffered saline (PBS) solution after incubation. Pre-aggregated Cy3-labeled Aβ42 (5 μg/mL) and DiI-Ac-LDL (5 μg/mL) were then added to the chamber slide, and incubated at 37°C for 2 h, in order to analyze microglial uptake. The cells were then washed several times with probe-free media, fixed with 3% formaldehyde in PBS, and observed *via* fluorescent microscopy. The fields used for quantification were selected randomly throughout the slide, and were focused with phase contrast optics prior to viewing the fluorescence. Cells were identified in the image using an intensity threshold set at the mean pixel intensity for the whole image, plus the standard deviation of the pixel intensity.

Immunofluorescence phagocytosis assay

Microglial phagocytosis activity was quantified by measuring the fluorescence of the fluorescently labeled Aβ42 and Ac-LDL, which were internalized by the microglia using the Phagocytosis Assay Kit (*Vybrant*™, Molecular Probes, U.S.A.). The cells were pre-treated with Rg3 for 1, 4, 8, 24, and 36 h, and then incubated with Cy3-Aβ42 and DiI-Ac-LDL for an additional 4 h at room temperature, after the cells had been rinsed three times. Trypan blue was added immediately after the removal of all reactants from the cells. The plate wells were then read by a *Tecan Genios Pro* at an excitation wavelength of 535 nm, and 612 nm emission.

RT-PCR and Western blots

In order to conduct the reverse transcription polymerase chain reaction (RT-PCR) of MSRA expression, the microglia were plated on six-well poly-D-lysine-coated plates, at a density of 1.5×10^5 cells/well in DMEM containing 1% FBS. The target products for PCR were 485 bp (sense, 5'-tag aca cgg gac gct tcc ag-3'; anti-sense, 5'-tcc tgg agc acc agg tgg ac-3') MSR-A and 186 bp (sense, 5'-tga ccg agc gtg gct aca gc-3'; anti-sense, 5'- acc gct cat tgc cga tag tg-3') β-actin. The PCR products amplified from the cDNA (30 cycles with annealing at 60°C for 20 s) were electrophoresed on 1.2% agarose gel. To ensure sequential results, we also performed Western blots. In brief, the cells were washed with PBS and lysed in lysis buffer containing protease- and phosphatase-inhibitors. The protein lysates were then assayed with a BCA protein assay kit (Sigma, U.S.A.). Then, 30 μg of protein lysates were boiled for 5 min, and electrophoresed on 10% SDS-polyacrylamide gel. The gel was transferred to a polyvinlidene fluoride (PVDF) membrane, which was blocked with tris-buffered saline/TWEEN-20 (TBST) containing 5% skim milk (blocking buffer), at room temperature for 1.5 h. The membrane was then incubated with anti-SR-A (MSRA) polyclonal antibody (Santa Cruz, U.S.A.). After washing with TBST for 15-5-5 min, the membrane was incubated with a secondary antibody, rabbit anti-goat IgG, conjugated with horseradish peroxidase (Zymed, U.S.A.), at room temperature for 2 h. The membrane was then washed in TBST buffer and developed with an enhanced chemiluminescence detection system (Amersham). Using the GraphPad Prizm 4 software, the data was analyzed as the mean±S.D. A P-value of <0.05 was considered to be statistically significant. The gel bands were assessed with the UVIDocMW program.

RESULTS

In order to characterize and quantify the uptake of Aβ in the microglia, rat primary microglial cultures were treated with Cy3-Aβ42. The fluorescence-labeled peptides were detected in the microglia, but the fucoidan-treated cells were proved to be incapable of binding with the peptides (Fig. 1B). This indicated that the microglia were capable of internalizing Cy3-Aβ through the MSRs. We then confirmed that our microglial cultures could both bind to and saturably internalize Aβ when treated with Rg3. Although the Aβ42 peptides were bound in small amounts to the normal non-treated microglia (Fig 1A), binding was observed to be greatly intensified in the presence of Rg3. This binding intensification reached its zenith at an incubation time of 4 h (Fig. 1, 2). These results were then compared with those generated with Ac-LDL, which shares the MSRs with Aβ (Paresce *et al.*, 1996, 1997).

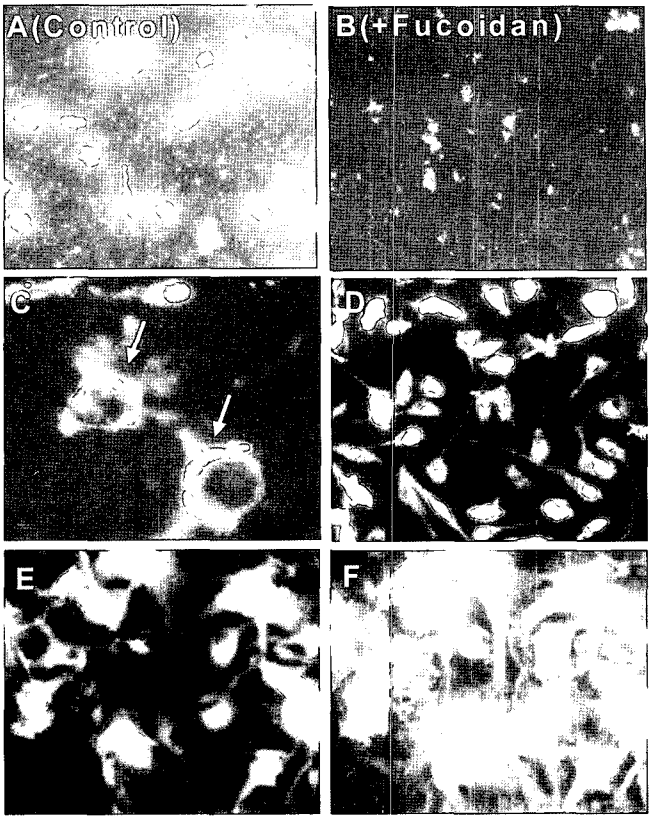

Fig. 1. Effects of Rg3 treatment on Cy3-Aβ uptake by microglia. After 4 h of pre-treatment with Rg3, the microglia were incubated with Cy3-Aβ42 (5 μg/mL) for 2 h. After each time-course, the cells were rinsed extensively, fixed, and imaged by digital fluorescence microscopy. The Cy3 fluorescence intensity remaining in each cell was seen on the digital images. Fluorescence (A-F) microscopic images of cells incubated with Cy3-Aβ42 are shown. Control (A) and fucoidan-treated cells (B) were then measured, in order to compare the degree of uptake between the Rg3-treated and the non-treated microglia. Clustered Cy3-Aβ42 on the cell surface (C) was measured (white arrow) after 1 h of Rg3 pre-treatment, and then the internalized Cy3-Aβ42 in the cells was saturably measured after 2 h of Rg3 pre-treatment. (D) and (E) was serial captures of different magnification (×100 and ×200, respectively) and (F) shows the extensively saturated cells with Cy3-Aβ42 after prolonged incubation for an extra 1 h with Cy3-Aβ42.

Cells incubated with 5 μg/mL Cy3-Aβ42 or 5 μg/mL DiI-Ac-LDL at 37°C for 2 h after 1-8 h of pre-treatment with Rg3 are shown in Fig. 2. We found that the Aβ42 and Ac-LDL peptides, when added to the culture, bound to the cell surface receptor (scavenging receptor), and we quantified this effect by measurements of average pixel intensity (Fig. 2). In this study, we used Ac-LDL to evaluate MSRA binding. We then assessed the effects of Rg3 on microglial uptake. We observed significant changes in fluorescent intensity, which reflected the amounts of internalized Cy3-Aβ or DiI-Ac-LDL in the microglia (Fig. 2). Our data also demonstrated that Rg3 played a very effective role in

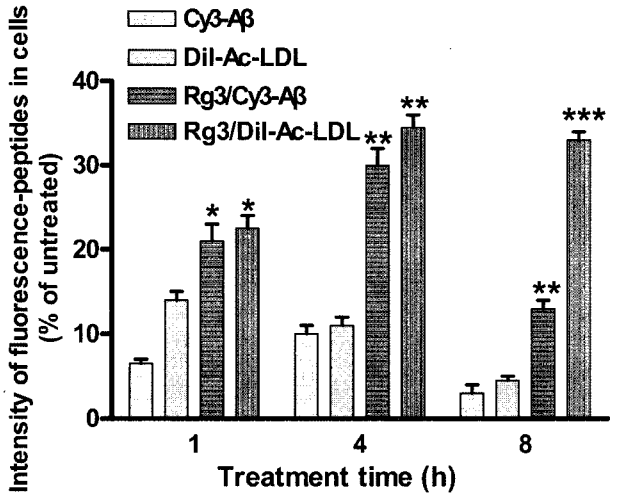

Fig. 2. Internalization of Aβ42 and Ac-LDL by the microglia is enhanced in the presence of Rg3. Microglia plated on 4-well chamber slides were loaded with Cy3-labeled Aβ42 (5 μg/mL) or DiI-labeled Ac-LDL (5 μg/mL) for 2 h after pre-treatment with 25 μg/mL of Rg3 for 1, 4, and 8 h. After each time-course, cells were rinsed, fixed, and imaged by fluorescence microscopy, as is described in the Materials and Methods. The total intensity of the fluorescence peptides in cells was measured as the sum of all intensity in each object identified as a cell. The data were expressed as the mean ± S.D. of a representative experiment repeated three times. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ for Rg3 plus Cy3-Aβ42 vs. Cy3-Aβ42 alone or Rg3 plus DiI-Ac-LDL vs. DiI-Ac-LDL alone.

enhancing microglial uptake, and that this enhancement was maximized at 4 h and persisted for up to 8 h (Fig. 2). As reported by Frackowiak *et al.* (1992), a small fraction of internalized Aβ and the extremely limited efficacy of the microglia with regard to the degradation of Aβ fibrils, might result in the blockage of microglial Aβ clearance. These issues warranted further examination, to determine whether Rg3 promotes microglial digestion or the clearance of internalized peptides to the exterior of the cells. We determined that the clearance of internalized Aβ from the microglia was enhanced in the presence of Rg3. Conversely, the Rg3-untreated microglia were not shown to clear the internalized Aβ very well, even with prolonged incubation times of up to 36 h (Fig. 3A). Interestingly, maximal microglial internalization occurred at 8 hours in the presence of Rg3, but at 4 h in the microglia which had not been treated with Rg3. This indicates that Rg3 may perform some as-yet-unidentified function in the promotion of microglial Aβ uptake. A similar, albeit less effective, Rg3 contributed to the uptake of Ac-LDL comparing to the Ac-LDL single group (Fig. 3B). We then attempted to ascertain whether MSRA expression could be activated by Rg3. The effect of Rg3 on the microglial expression of the MSRA gene appeared to be both dose- and time-dependent, and reached a maximal value of 25 μg/mL after 4 h of incubation (Fig. 4A). In order to confirm whether MSRA gene expression could be amplified by Rg3 treatment, we analyzed the levels of microglial MSRA proteins via Western blotting, which generated dose-dependent results, consistent with the results of RT-PCR, after 4 h of incubation (Fig. 4B). The presence of activated microglia within the amyloid plaques may indicate that they are involved in the phagocytosis of amyloid fibrils or aggregates, thereby contributing to their clearance. However, the presence of activated microglia in AD brains may also be associated with some deleterious effects, namely that they may directly contribute to amyloidosis by participating in the formation of Aβ, as well as the generation of proinflammatory and potentially cytotoxic mediators which induce chronic inflammation (Paresce *et al.*, 1996). As those negative effects may be triggered by an overproduction of Aβ fibrils, the effective microglial clearance of fibrils during earlier phases

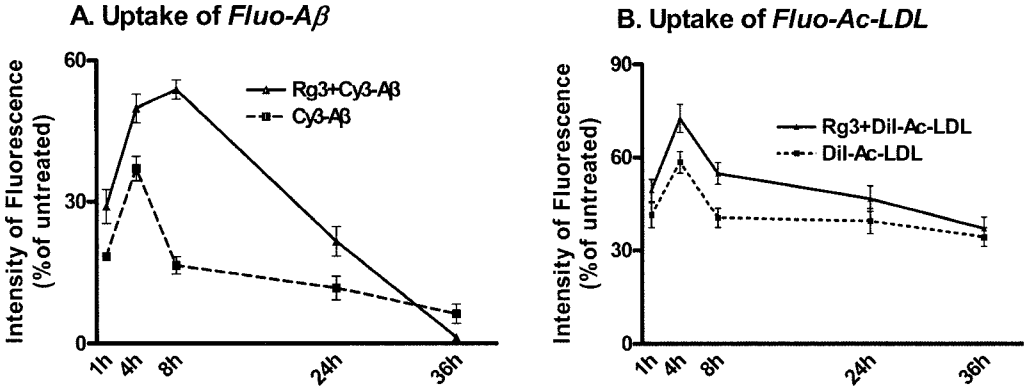

Fig. 3. Time-effect relationship of Rg3 on the microglial internalization of Cy3-Aβ42 and DiI-Ac-LDL. Cells were cultured in 96-well plates, treated with Rg3 for 1, 4, 8, 24, and 36 h, and then incubated with Cy3-Aβ42 (A) and DiI-Ac-LDL (B) at room temperature for 4 h. Cells were then rinsed with PBS, and 100 mL of trypan blue was added to quench any extra cellular fluorescence. The intensity of fluorescence of internalized Cy3-Aβ42 and DiI-Ac-LDL were determined on a fluorescence microplate reader. Data are expressed as mean ± S.D. (lines) values of triplicate determinations.

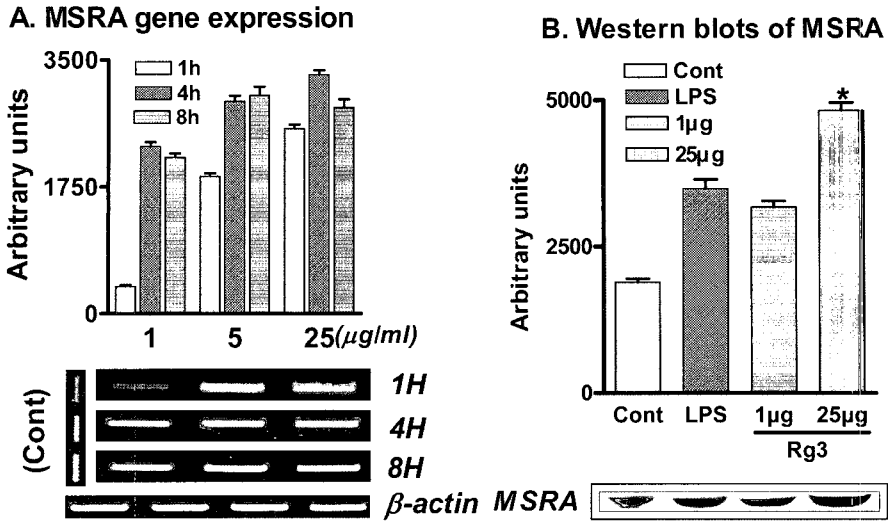

Fig. 4. MSRA expression by rat microglia in response to Rg3 (dose- and time-effect relationship between RT-PCR and Western blots). (A) Cells were plated on 6-well plates, treated with Rg3 (1, 5, and 25 μg/mL) for 1, 4, or 8 h, and the extracted mRNAs were amplified *via* RT-PCR. The non-treated control was used as normal and β-actin as standard controls, respectively. (B) The cell lysates from the cytosol treated for 4 h with Rg3 (1 or 25 μg/mL) were then analyzed by Western blot, using anti-MSRA antibody. Lipopolysaccharides (5 μg/mL) were used as a positive control for Rg3. The data represent the mean ± S.D. of triplicate determinations (*p<0.05 vs. lipopolysaccharide; B).

of AD development may constitute one of the keys to the effective prevention of AD. Additionally, MSR-mediated Aβ uptake has been reported to play a role in the clearance of Aβ, which is continuously generated in both normal and AD brains (McGeer *et al.*, 1992). Additionally, it appears probable that MSRA is the MSR which plays the more significant role in Aβ clearance in the brain, although the loss of MSR can be compensated for by the presence of other MSRs, including FPR, and RAGE (Paresce *et al.*, 1996; Rogers *et al.*, 2002). Based on our overall results, it can be surmised that Rg3 is promising candidate for the enhancement of microglial Aβ clearance. This phenomenon may be attributable to the Rg3-mediated enhancement of MSRA expression (Fig. 4A-B). In particular, the reduction of Aβ clearance and the slow degradation of Aβ aggregates, both of which result in the formation of amyloid plaques in AD brains (Paresce *et al.*, 1997; Chung *et al.*, 1999) may be effectively ameliorated *via* Rg3 treatment, due to the resultant enhancements in MSRA expression and the acceleration of Aβ uptake, coupled with the enhanced clearance of Aβ from the cells.

DISCUSSION

Based upon the results on primary rat microglia for phagocytosis and the expression of MSRA, we found that ginsenoside Rg3 may be a potential effector in AD brain marked with overproduced Aβ and with amyloid plaque (so called senile plaque) and the mechanism may be due to its role in promoting the microglial uptake of Aβ along with the stimulation of MSRA expression. Moreover, the internalized Aβ was more effectively digested from microglia in the presence of Rg3. More interesting finding was that Rg3 may accelerate the microglial phagocytosis of Aβ at 4 h incubation time, which was maximized at 8 h, and the internalized Aβ was almost clearly digested within 36 h. Taken together, these findings have important implications for the therapeutic use of Rg3 in the treatment and the prevention of AD. However, whether the released Aβ from microglia is effectively degraded into non-toxic forms or is released in an intact form of Aβ itself that is known to be associated with amyloid plaque still remains to be more clearly elucidated, though the internalized Aβ is effectively digested when treated with Rg3.

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