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# BiP/GRP78-Induced Production of Cytokines and Uptake of Amyloid-$\beta$(1-42) Peptide in Microglia

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**In the brains of Alzheimer's disease (AD) patients, fibrillar amyloid-$\beta$ peptides (A$\beta$) are markedly accumulated and the microglia associate with the amyloid plaques. However, the regulation of A$\beta$ clearance is still unclear. In the present study, we examined the effect of a chaperone protein BiP/GRP78 on the microglial function. Exogenous addition of recombinant BiP/GRP78 induced the production of cytokines such as interleukin-6 and tumor necrosis factor-$\alpha$, but heat treatment of this protein abolished the activity. Although A$\beta$(1-42) did not induce cytokine production, it was taken up by the microglia. In addition, the amount of A$\beta$(1-42) uptake and the number of microglia that phagocytosed A$\beta$(1-42) were markedly increased by BiP/GRP78. Exogenous BiP/GRP78 also translocated to the endoplasmic reticulum (ER). These results suggest that BiP/GRP78 stimulates A$\beta$ clearance in the microglia, and that dysfunction in the ER may cause the accumulation of extracellular A$\beta$(1-42).** 

***Key Words:*** **microglia; amyloid-$\beta$; clearance; BiP (GRP78); cytokine; Alzheimer's disease.**

The immunoglobulin heavy chain-binding protein (BiP, also known as a 78-kDa of glucose-regulated protein GRP78) is induced in the endoplasmic reticulum (ER) by stressful condition (1). The ER stress activates the signaling pathway of unfolded protein response (UPR) involving ER-resident transmembrane kinases, IRE1 and PERK (2). Recent studies suggest that ER stress-induced apoptosis is mediated by activation of caspase-12 which is localized in the ER (3) or c-Jun N-terminal kinase which is associated with IRE1 and TRAF2 (4). It is known that BiP/GRP78 acts as a molecular chaperone in the ER for naive, aberrantly folded or mutated proteins (1). Therefore, UPR-induced BiP/GRP78 may operate to alleviate the ER stress and/or to prevent apoptosis (1). However, other functions of BiP/GRP78 are unknown.

One hallmark of Alzheimer's disease (AD) is the accumulation of fibrillar amyloid-$\beta$ peptides (A$\beta$), these aggregations and the formation of plaques in the brain (5). Therefore, it is important to understand the balance of production and clearance of A$\beta$. However, the regulation of A$\beta$ clearance is still unclear. Missense mutations in the presenilin-1 (PS1) gene, that link to some early-onset cases of the familial Alzheimer's disease (FAD), increase the production of A$\beta$(1-42) (6), the vulnerability to neuronal apoptosis through an increase of ER-mediated $Ca^{2+}$ release (7), and the down-regulation of the UPR signaling (8). These observations suggest that dysfunction in the ER may participate in the pathogenesis of AD brains. In the present study, we examined effects of BiP/GRP78 on the cytokine production and A$\beta$ phagocytosis in the microglia.

## MATERIALS AND METHODS

*Microglial culture and cell treatment.* Mixed glial cells (mixture of astrocytes and microglia) were prepared from cerebral hemispheres of newborn Wistar rats. Tissue suspension was filtered through 50-$\mu$m diameter nylon mesh (cell strainers, Falcon) into 50-ml tubes and cells were collected by centrifugation at 200*g* for 10 min. Cells were resuspended in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, plated in 100-mm diameter dishes and incubated at 37°C in 5% $CO_2$/95% air. Subsequently, floating microglia were harvested from mixed glial cultures and plated in new culture dishes. In the process of preparation and

Abbreviations used: A$\beta$, amyloid-$\beta$ peptide; AD, Alzheimer's disease; BiP, immunoglobulin heavy chain-binding protein; ER, endoplasmic reticulum; EOR, ER overload response; FAD, familial Alzheimer's disease; GRP, glucose-regulated protein; IL-6, interleukin-6; PS1, presenilin-1; TNF-$\alpha$, tumor necrosis factor-$\alpha$; UPR, unfolded protein response.

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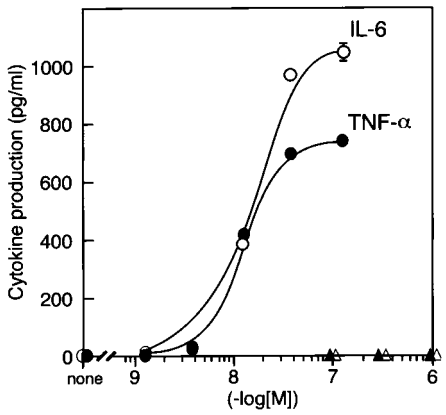


**FIG. 1.** BiP/GRP78-induced production of cytokines from microglia. Isolated microglia were treated with recombinant BiP/GRP78 (○, ●) or Aβ(1-42) (△, ▲). After 24 h, supernatant from each well was recovered and the amount of IL-6 (○, △) or TNF-α (●, ▲) was determined by ELISA. Each value is the mean ± SEM of three determinations.

culture, extreme care was taken to avoid contamination of endotoxins (9, 10). Pure microglia thus obtained (over 97%) were treated with recombinant BiP/GRP78 (at 0–128 nM, StressGen, Victoria, BC, Canada) in the presence or absence of Aβ(1-42) (at 0–1 μM, AnaSpec, San Jose, CA).

*Assay of cytokine production.* The supernatant from each well was recovered and the amount of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) released from the microglia was determined by the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (BioSource, Camarillo, CA).

*Assay of Aβ-binding/phagocytosis.* Treated microglia were collected and lysed, and then samples were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE; 20% polyacrylamide gels) in Tris–Tricine buffer. After an immunoblotting of anti-Aβ antibody (Chemicon, Temecula, CA), the detection of Aβ oligomers was performed by a chemiluminescence assay (ECL kit, Amersham Pharmacia Biotech, Buckinghamshire, UK). For quantitative analysis of total amount in Aβ oligomers, the X-ray film was scanned with a CCD color scanner (DuoScan, AGFA), and then the density was measured. The data in the densitometric analysis is given as mean ± standard error of mean (SEM). Statistical significance of differences was determined by an analysis of variance (ANOVA). Further statistical analysis for post hoc comparisons was done by the Bonferroni/Dunn test.

*Confocal microscopy.* After treatment with exogenous BiP/GRP78 in the presence or absence of Aβ(1-42), the microglia were immediately fixed and then incubated for 4 days at 4°C with both mouse anti-CD11b antibody and rabbit anti-Aβ antibody, or with rabbit anti-BiP/GRP78 antibody. The labeled primary antibodies were detected by FITC-anti-mouse IgG antibody and rhodamine-anti-rabbit IgG antibody, and then these fluorescences were observed with a laser scanning confocal microscope LSM410 (Carl Zeiss, Germany).

## RESULTS

### *BiP/GRP78-Induced Production of Cytokines*

We preliminarily found that the expression level of BiP/GRP78 protein in the microglia was lower than that in the neurons (data not shown). Therefore, we exogenously added recombinant BiP/GRP78 protein into a culture of isolated microglia. Exogenous BiP/GRP78 (at less than 0.1 μM) induced production of IL-6 and TNF-α in a concentration-dependent manner (Fig. 1). The amount of IL-6 production was higher than that of TNF-α. To exclude a possibility of endotoxin contamination, recombinant BiP/GRP78 sample was heat treated at 100°C for 20 min before incubation with the microglia. Heat treatment completely abolished the ability of BiP/GRP78 to cytokine production (data not shown), suggesting that the observed activity of the BiP/GRP78 was not due to endotoxin contamination. On the other hand, Aβ(1-42), non-Aβ component of AD amyloid (NAC) or HSP27 even at 10 μM did not induce production of these cytokines (data not shown).

### *BiP/GRP78-Induced Enhancement of Aβ Uptake and Their Localization*

Aβ(1-42) was bound and/or phagocytosed to the microglia in a concentration dependent manner (Fig. 2).

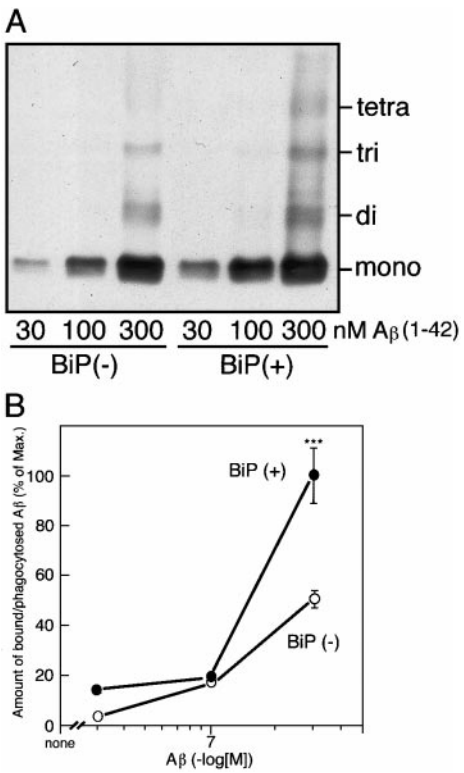


**FIG. 2.** BiP/GRP78-induced enhancement of bound and/or phagocytosed Aβ(1-42) to microglia. Microglia were incubated with Aβ(1-42) at 30–300 nM in the presence (●) or absence (○) of 64 nM BiP/GRP78. After 24 h, treated microglia were collected and lysed. Subsequently, samples were subjected to immunoblotting of anti-Aβ antibody. (A) Oligomers such as mono-, di-, tri- and tetramer of Aβ(1-42) were detected. (B) Total amount of these oligomers of bound/phagocytosed Aβ was measured by densitometric analysis. Each value is the mean ± SEM of three determinations. ***$P < 0.001$ versus the value without BiP/GRP78.

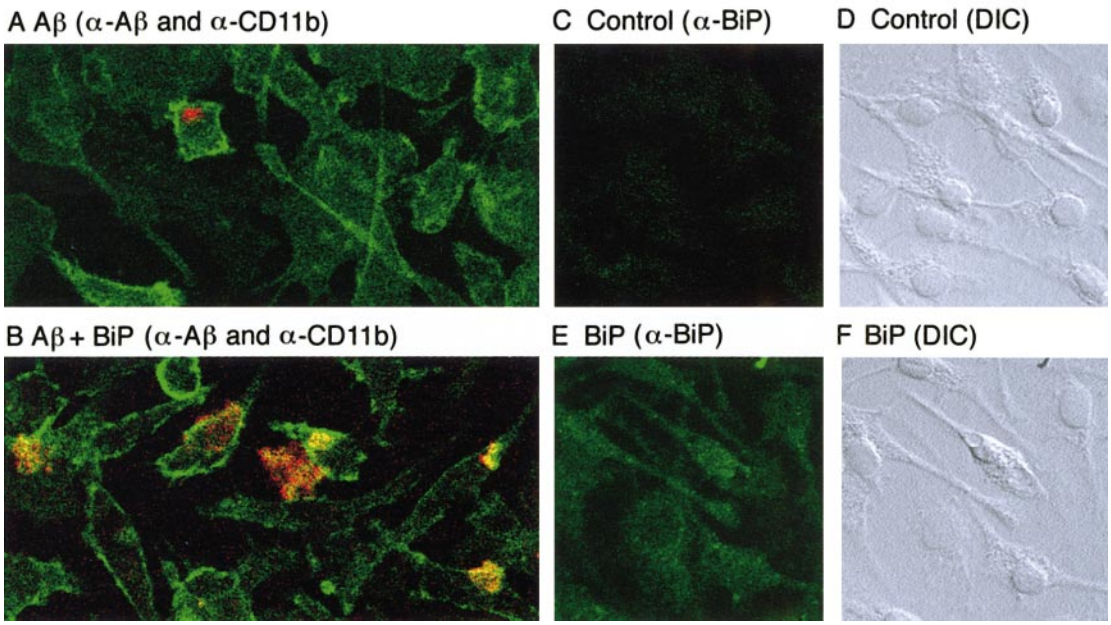


**FIG. 3.** Confocal analysis of A$\beta$(1-42) phagocytosis and BiP/GRP78 localization. (A and B) Microglia were incubated with 1 $\mu$M A$\beta$(1-42) in the presence of vehicle (A) or 64 nM BiP/GRP78 (B). After 24 h, treated microglia were immediately fixed and co-incubated with mouse anti-CD11b antibody and rabbit anti-A$\beta$ antibody. Phagocytosed A$\beta$ (red) were detected in the vesicles of microglia that intensively expressed membrane protein CD11b (green). Exogenous BiP/GRP78 induced increase in the number of microglia that phagocytosed A$\beta$(1-42). (C-D) After treatment for 60 min with vehicle (C, D) or 64 nM BiP/GRP78 (E, F), microglia were labeled by anti-BiP/GRP78 antibody. Immunoreactivity of BiP/GRP78 (green) was markedly detected in the perinuclear region and cytoplasm (E). Images (the difference interference contrast, DIC) were obtained by confocal laser microscopy (D, F). These data represented three independent experiments with similar results.

In this case, oligomers (such as dimer, trimer, tetramer, etc.) of A$\beta$(1-42) were detected by an immunoblotting of anti-A$\beta$ antibody even in the presence of a potent detergent SDS (Fig. 2A). Thus, soluble and aggregated A$\beta$(1-42) might be bound and/or phagocytosed to the microglia. By the laser confocal microscopy, the immunoreactivity of A$\beta$ was detected in the small vesicles in few microglia that expressed membrane protein CD11b (also known as the complement receptor-3 or C3biR) (Fig. 3). Exogenous BiP/GRP78 significantly increased the amount of A$\beta$(1-42) uptake in the microglia (Fig. 2B) and the number of microglia that phagocytosed A$\beta$(1-42) (Fig. 3B). Thus, exogenous BiP/GRP78 at lower concentration induced microglial activation and significantly enhanced A$\beta$ clearance. On the other hand, exogenous addition of recombinant protein intensively increased the immunoreactivity of BiP/GRP78 in the perinuclear region and cytoplasm (Fig. 3E), similar to immunoreactivities of PS1 and Ire1 that are localized in the ER (8).

## DISCUSSION

Previous studies have found that the production of nitric oxide or cytokines from microglia or monocytes by A$\beta$(1-42) was necessary to the additional stimulant such as interferon-$\gamma$ or lipopolysaccharide (11, 12). Our result also indicated A$\beta$(1-42) alone did not induce production of IL-6 and TNF-$\alpha$. In contrast, A$\beta$(1-42) at lower concentration (30–300 nM) was taken up by the microglia. Since oligomers of A$\beta$(1-42) were also detected by immunoblot analysis, soluble and aggregated A$\beta$(1-42) may be phagocytosed to the microglia. Recombinant BiP/GRP78 induced cytokine production from the microglia, but its heat treatment abolished the activity. Although IL-6 and TNF-$\alpha$ are known as proinflammatory cytokines in the immune system, these cytokines may act as autocrine and paracrine mediators that induce glial proliferation and neuroprotective response in the brain (13, 14, 15). BiP/GRP78 itself may induce microglial activation. In addition, the uptake of A$\beta$(1-42) was significantly enhanced by exogenous BiP/GRP78. Although the relationship between cytokine production and A$\beta$ uptake was not clear, we obtained preliminary result that IL-6 or TNF-$\alpha$ did not markedly increase A$\beta$ uptake suggesting that BiP/GRP78-induced A$\beta$ uptake may not be mediated by IL-6 or TNF-$\alpha$. Since it is known that ER overload response (EOR) induces NF-$\kappa$B activation and then produces cytokines (1), exogenous BiP/GRP78 may activate EOR in the microglia. On the other hand, it is unknown whether BiP/GRP78 is extracellularly

secreted *in vivo* brain, but it is known that GRP94-like protein associated with cholesterol esterase is secreted from the pancreas (16). Since BiP/GRP78 protein was constitutively and highly expressed in the neurons in comparison with that in the glial cells *in vivo* brain (unpublished observation), we speculated that BiP/GRP78 protein may be extracellularly leaked from damaged, broken or dead neurons caused by neurodegeneration.

It is reported that A$\beta$ clearance in the microglia is mediated by membrane receptors such as scavenger receptors (17, 18). More recently, anti-A$\beta$ antibodies that were produced by A$\beta$ vaccine therapy induced Fc receptor-mediated phagocytosis of A$\beta$ (19, 20). In the present study, exogenous BiP/GRP78 intensively increased its immunoreactivity in the perinuclear region and cytoplasm. Since BiP/GRP78 involves the carboxyl terminal sequence Lys-Asp-Glu-Leu (KDEL) that is the ER-retrieval signal, exogenous BiP/GRP78 may translocate to the ER and then facilitate A$\beta$ phagocytosis. Thus, BiP/GRP78-induced clearance of A$\beta$ is mediated by a different mechanism compared to the clearance by anti-A$\beta$ antibodies.

Katayama *et al.* recently reported that expression level of BiP/GRP78 protein was reduced slightly in brains of patients with sporadic AD and markedly in PS1-mutant FAD brains and that overexpression of BiP/GRP78 inhibited ER stress-induced death of neuronal cells (8). Thus, BiP/GRP78 differently affects to the neurons and microglia such as neuroprotection and A$\beta$ clearance, respectively. In addition, since BiP/GRP78 expression was lower level in AD and FAD brains, BiP/GRP78-induced A$\beta$ clearance may be downregulated and results in the accumulation of extracellular A$\beta$.

In conclusion, extracellular A$\beta$(1-42) was usually taken up by the microglia. BiP/GRP78 induced production of cytokines and enhanced phagocytosis of A$\beta$(1-42) in the microglia. Thus, we have identified novel biological functions exerted by BiP/GRP78 that induced microglial activation and stimulated A$\beta$ clearance. BiP/GRP78 may be another microglial activator for the increase of A$\beta$ clearance besides anti-A$\beta$ antibodies.

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