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GM1 up-regulates Ubiquilin 1 expression in human neuroblastoma cells and rat cortical neurons

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Abstract

GM1 ganglioside was reported to mediate the amyloid β-protein (Aβ) secretion and accumulation in the pathogenesis of Alzheimer's disease (AD). The objective of this project was to comprehend the underlying molecular changes related to amyloid β-protein precursor (APP) processing pathway induced by GM1. Using suppression subtractive hybridisation (SSH), we detected one prominent sequence with increased expression in human neuroblastoma cells that stably transfected with human APP695 cDNA treated with GM1. This transcript has high identity to human Ubiquilin 1 gene. Differential expression was initially confirmed by dot blot hybridization. This result was further authenticated with quantitative real-time polymerase chain reaction (RT-PCR) analysis. Furthermore, using Western blots, we discovered that GM1 stimulated the expression of Ubiquilin 1 in human neuroblastoma cells and rat cortical neurons while other gangliosides Asialo-GM1 and GD1b did not. Ubiquilin 1 is one of the candidate genes of AD, which have been shown to modulate the γ-secretase components in the proteolytic processing of APP, and is therefore a putative candidate for further investigation of GM1 mechanisms in the etiology and pathology of AD.

Keywords: Ubiquilin 1; GM1 ganglioside; Alzheimer's disease; Gene expression; Suppression subtractive hybridization

The presence of plaques containing amyloid β-protein (Aβ) is a pathological hallmark in diagnosing Alzheimer's disease (AD) [5,11]. Aβ is generated from the Alzheimer amyloid β-protein precursor (APP) by the two consecutive cuts of two proteases, known as β-secretase (BACE) and γ-secretase [9,12]. Consequently, factors acting on the processing pathways of APP are very intriguing study targets to unravel the etiology and therapy of AD.

GM1 ganglioside is a sialic acid containing the glycosphingolipid found in the plasma membrane of most vertebrate cells, particularly in the nervous system. Recently, an increasing number of surveys showed that GM1 ganglioside plays an important role in the amyloidogenic processing of AD. Evidence from *in vivo* studies has shown that GM1 acts as a seed for the polymerization of Aβ, and its subsequent binding to Aβ induces the formation of diffuse plaques and amyloid fibrils [10,22]. Moreover, GM1 strongly participates in the release of Aβ from the membranes, and as such participates in amyloid precipitation [20]. In our previous studies, we demonstrated that GM1 regulated the proteolysis of APP, and stimulated Aβ production significantly in cultured cells [24]. Tamboli et al. [19] also demonstrated that inhibition of glycosphingolipid biosynthesis reduces the secretion levels of the APP and Aβ. However, the precise role of GM1 in the proteolytic processing of APP and Aβ production is not well understood.

There are several studies on the gene expression associated with GM1. GM1 can stimulate the expression of interleukin-1 beta, nerve growth factor, epidermal growth factor and platelet-derived growth factor receptor and so on [7,16,18]. However, there are no comprehensive profiles of differentially expressed genes by GM1 before. In order to identify differentially expressed transcripts related to APP processing pathways in response to exogenous ganglioside GM1, including low-abundance genes, we utilized suppression subtractive hybridization (SSH) techniques [6]. SSH provides a comprehensive profile of differentially expressed genes and can isolate low-abundance genes that may avoid detection by microarray

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analysis [3]. We detected one prominent sequence in the GM1-treated cells in at least three clones that has a high sequence identity with human Ubiquilin 1 gene, which was reported as an intriguing candidate gene of AD [2]. We have completed a preliminary validation of this candidate gene, and confirmed its enhanced expression in the GM1-treated SH-SY5Y cells, and in rat cortical neurons treated with exogenous GM1.

Mixed cortical neurons were prepared from 14-day old fetal rats, as described previously [17]. SH-SY5Y cells stably transfected with APP695 cDNA (SH-SY5Y APP695) were cultivated as described previously [24]. Ganglioside GM1, Asiolo-GM1 and GD1b were from Sigma, Saint Louis, MO. According to our previous work with GM1 [24], we treated the experimental cells with 1–50 μM gangliosides for 8 h in conditioned medium containing no FCS.

In order to identify genes up-regulated by GM1, we used the cDNA from SH-SY5Y APP695 cells treated with 50 μM GM1 as the tester cDNA, and the cDNA from untreated cells as the driver cDNA. Polyadenylated RNA was isolated according to the manufacturer's instructions of the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the Oligotex kit (Qiagen, Valencia, CA, USA). The subtracted cDNA library was constructed using a PCR-SelectTM cDNA Subtraction kit (Clontech, Palo Alto, CA, USA), according to the manufacturer's instructions.

The subtracted library subsequently was cloned directly into a pGEM-T vector according to the technical manual of the vector system (Promega, Madison, WI, USA). The library was plated on agar plates and 336 individual clones were amplified with PCR using T3 and T7 primers. The PCR products were denatured in an equal volume and dot blotted in duplicate onto the nylon membranes (Schleicher & Schuell, Keene, NH, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The tester and driver probes labeled with digoxigenin-UTP were generated from 50 μM GM1-treated and untreated SH-SY5Y APP695 cRNA preparations according to the manufacturer's protocol (Roche Diagnostics GmbH, Germany). The processed membranes were hybridized with tester probe first and re-hybridized with driver the probe. The results were quantified using Quantity One software 4.1 (Bio-Rad Laboratories, Hercules, CA, USA), and the values were expressed as a ratio of the candidate gene to GAPDH. Differentially expressed clones were sequenced using a DNA sequencer (CEQ2000XL, Beckman-Coulter, Fullerton, CA, USA). The resulting sequence data were identified by searching for known homologous sequences and expressed sequence tags present in the National Centre for Biotechnology Information (NCBI) database.

To further confirm the differential expression of the transcript discovered by SSH, quantitative real-time polymerase chain (RT-PCR) analysis was next performed. SH-SY5Y APP695 cells were treated with GM1 at various concentrations (0, 1, 10 or 50 μM) for 8 h and total RNA was isolated using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCRs were conducted in real-time PCR detection system (Bioer, Line-Gene) with a Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA, USA). GAPDH and β-actin were used as control genes. The primers used for quantitative RT-PCR were: Ubiquilin 1, 5′-TAC CAG TAG TGC CAC CCA CA-3′ (forward) and 5′-ATC CAT TTG CGG CCT TGG TG-3′ (reverse); GAPDH, 5′-GAG TCA ACG GAT TTG GTC GT-3′ (forward) and 5′-GAC AAG CTT CCC GTT CTC AG-3′ (reverse); β-actin, AGA GCT ACG AGC TGC CTG AC (forward) and 5′-AGC ACT GTG TTG GCG TAC AG-3′ (reverse). Changes in fluorescence were recorded as the temperature was increased from 65 to 95 °C at a rate of 0.2 °C/s to obtain a DNA melting curve.

Ubiquilin 1 protein levels were analyzed by Western blotting of cell extracts from SH-SY5Y APP695 cells and rat mixed cortical neurons pretreated with different gangliosides concentrations (0, 1, 10 or 50 μM) for 8 h [14]. Primary antibodies used were mouse anti-Ubiquilin (Zymed Laboratories, South San Francisco, CA, USA) and mouse anti-GAPDH (KangChen, Shanghai, China). Detection was made by ECL method (Amersham Bioscience, NJ, USA). The band images were acquired using a CCD camera (Bio-Rad Laboratories, Hercules, CA, USA), and the band intensity was calculated with Quantity One 4.1 applied software.

Significant differences between the two groups were determined with Student's *t*-tests, and those among multiple groups were assessed by analysis of variance (ANOVA) and Fisher's protected least significant distance *post hoc* tests. A level of 0.05 ($P < 0.05$) was considered to be statistically significant.

Following SSH between GM1-treated and untreated cDNA samples, the differentially expressed PCR products were ligated into a pGEM-T vector and cloned into DH5α cells to construct the subtracted cDNA libraries. 336 cDNA clones were randomly selected from the subtracted cDNA libraries and re-screened for differential gene expression by dot blot hybridization. Partial clones were represented in Fig. 1. Sequence analyses showed that one up-regulated transcript has identity to human Ubiquilin 1 gene, which is recently reported to be an intriguing candidate gene of AD [1,2,15], being presented in at least three clones (Fig. 1). Thus, we selected this sequence for further validation.

We next performed quantitative RT-PCR analysis to further confirm the differential expression of Ubiquilin 1. The melting curve analysis illustrated a single peak indicative of a single amplification product (data not shown). Fig. 2 showed that

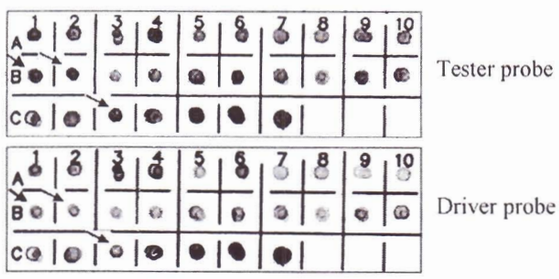

Fig. 1. An up-regulated transcript with high identity to human Ubiquilin 1 was presented by method of dot blot hybridization. Positive clones were chosen from the subtracted cDNA libraries and dot blotted onto nylon membranes, hybridized with digoxigenin-UTP labelled tester probe and re-hybridized with driver probe (from the GM1-treated and untreated SH-SY5Y APP695 cells, respectively). The three dots marked by arrows were clones have high identity to Ubiquilin 1. The last three dots were GAPDH.

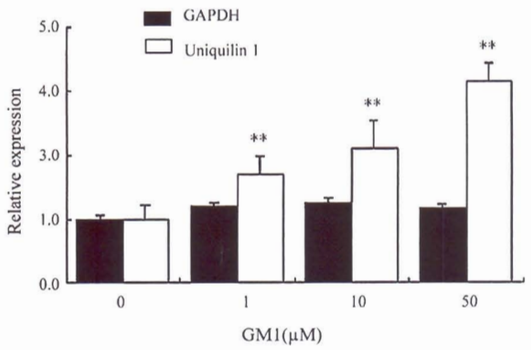

Fig. 2. Quantitative analysis of Ubiquilin 1 mRNA in SH-SY5Y APP695 cells treated with different concentrations of GM1. Total RNA was prepared from SH-SY5Y APP695 cells treated with different concentrations of GM1 (0, 1, 10 or 50 μM) for 8 h. Expression of Ubiquilin 1 and GAPDH were calculated by quantitative RT-PCR. Data in all lanes are normalized to that in lane 1 (expression absence of GM1). Values represent the mean ± S.E. ANOVA followed by Fisher's protected least significant distance *post hoc* tests were used to analyze the results. $n = 6$. $^{**}P < 0.01$ vs. absence of GM1.

GM1 treatment induced Ubiquilin 1 mRNA expression in a dose-dependent manner. Ubiquilin 1 increased slightly when the cells were treated with 1 μM GM1. In the presence of 50 μM GM1, Ubiquilin 1 increased by roughly 3-fold above that in the untreated cells. GAPDH was expressed consistently in range of 0–50 μM GM1 (Fig. 2). Similar significant results were obtained when the signal was related to β-actin control gene (data not shown).

The levels of Ubiquilin 1 protein in the GM1-treated SH-SY5Y APP695 cells and the rat mixed cortical neurons were investigated using Western blot analysis. Figs. 3 and 4 illustrated that in both of these cell types, GM1 dose-dependently increased Ubiquilin 1 protein levels. There are no significant differences between SH-SY5Y neuroblastoma cells and rat cortical neurons.

To detect the effects of other gangliosides, we examined the activation of Ubiquilin 1 in SH-SY5Y APP695 cells treated with Asialo-GM1 and GD1b using Western blot. GAPDH was used as the control. We demonstrated that the expressions of Ubiquilin 1 were not significantly changed by exposure to Asialo-GM1 and GD1b (Fig. 5).

Taken together, our results showed that GM1 up-regulates Ubiquilin 1 expression in human SH-SY5Y neuroblastoma cells and rat cortical neurons.

Alzheimer's disease is by far the most common cause of dementia. The pathological mechanisms involved in AD are intricate, and are not yet fully understood. Many researchers reported that GM1 ganglioside plays an important role on the pathology of AD and APP processing pathways [21,24]. But it is not clear that whether APP promotes the underlying molecular changes upon GM1 treatment. Therefore, we used APP transfected SH-SY5Y cell lines instead of untransfected SH-SY5Y cells. We showed previously that exogenous GM1 in the range of 1–50 μM confers no cellular toxicity, as assessed by measuring the release of lactate dehydrogenase into the culture media of SH-SY5Y APP695 cells [24]. Therefore, the altered expression of identified genes must have resulted from the direct effect of

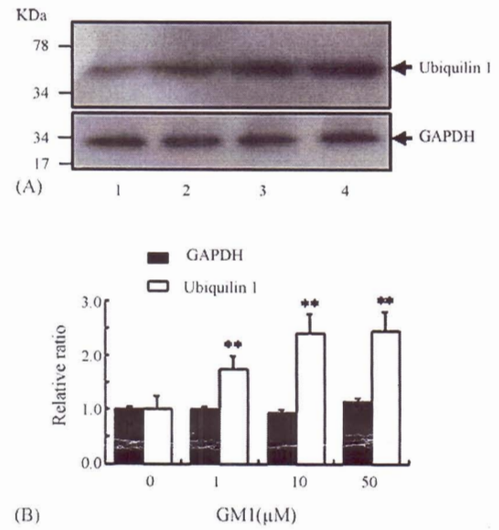

Fig. 3. Effects of GM1 ganglioside on Ubiquilin 1 expression levels in SH-SY5Y APP695 cells. (A) SH-SY5Y APP695 cells were cultured in the presence of 0, 1, 10 or 50 μM GM1 (lanes 1–4) for 8 h. Ubiquilin 1 levels in the lysates were detected by Western blot and GAPDH was used as the control. (B) quantification of Ubiquilin 1 and GAPDH were based on three independent experiments. Values are mean ± S.E. Statistical analysis of the results was carried out using ANOVA followed by Fisher's protected least significant distance *post hoc* test. $^{**}P < 0.01$ vs. 0 μM GM1 in lane 1.

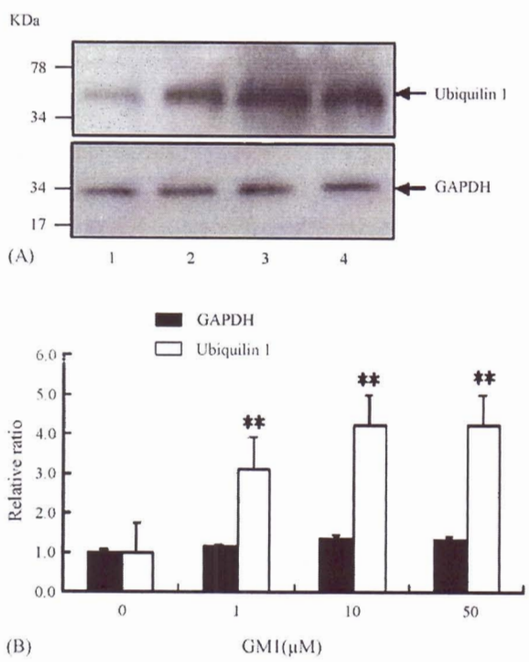

Fig. 4. Effects of GM1 ganglioside on Ubiquilin 1 expression levels in rat mixed cortical neurons. (A) Rat mixed cortical neurons were cultured in the presence of 0, 1, 10 or 50 μM GM1 (lanes 1–4) for 8 h. Ubiquilin 1 levels in the lysates were detected after GM1 incubation and GAPDH was used as the control. (B) Illustrates the quantitative analysis of Western blots. Values are mean ± S.E. $n = 3$. $^{**}P < 0.01$ vs. 0 μM GM1 in lane 1.

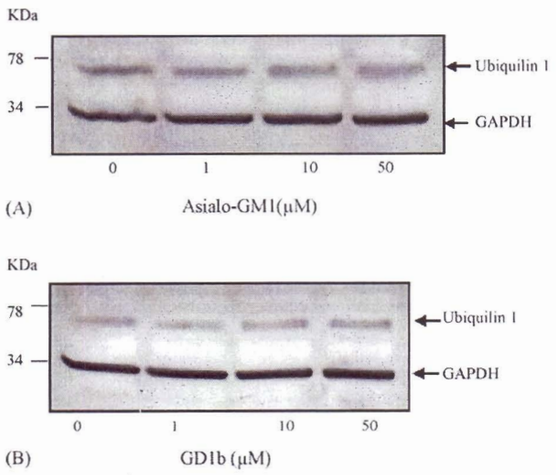

Fig. 5. Activation of Ubiquilin 1 by Asialo-GM1 and GD1b in SH-SY5Y APP695 cells. SH-SY5Y APP695 cells were exposed to Asialo-GM1 (A) and GD1b (B) of 0, 1, 10 or 50 μM for 8 h. Ubiquilin 1 and GAPDH levels in the lysates were detected by Western blot after incubation. Experiments were repeated three times.

GM1 itself, rather than from its indirect cytotoxicity. Herein, we detected one prominent sequence in at least three clones, which showed high identity to human gene Ubiquilin 1.

Ubiquilin 1 was reported as the new candidate gene of AD besides APP, presenilins (PS) and Apolipoprotein E [2]. Moreover, Ubiquilin 1 is highly expressed in human neurons, and is associated with neurofibrillary tangles (NFTs), which are the physiological hallmarks of AD [13]. Ubiquilin 1 also promotes the accumulation of PS *in vitro* [13]. PS is believed to constitute the core catalytic enzyme activity of γ-secretase, which is responsible for cleaving the APP to release Aβ [8,23]. Consistent with this observation is the finding that Ubiquilin 1 increases the synthesis of PS, and also affects other components of the γ-secretase complex, including Pen-2 and nicastrin components [14,15]. Intriguingly, Bertram et al. [1] reported in a clinical study, a strong linkage of variants in the Ubiquilin 1 gene in independent families with late-onset AD. These studies indicate that Ubiquilin 1 is likely to be relevant to certain aspects of AD onset and progression, and is capable of regulating the γ-secretase complex. Together with our previous work on ganglioside GM1 regulation of the proteolytic processing of APP via γ-secretase activity, we selected this sequence as our first candidate for validation.

This study enabled us to obtain a good preliminary validation of the increased expression of Ubiquilin 1. Differential gene expression was confirmed initially by dot blot hybridization. These results were further authenticated by quantitative RT-PCR, whereby GM1 increased the expression of Ubiquilin 1 mRNA in a dose-dependent manner. We also characterized the expression of Ubiquilin 1 by Western blots and showed that the protein expression level of Ubiquilin 1 was significantly induced by exogenous GM1 in a dose-dependent manner in SH-SY5Y APP695 cells and rat mixed cortical neurons. Therefore, we predict that the increased expression of Ubiquilin 1 may be involved in the mechanism of GM1-associated APP proteolytic processing pathways. To eliminate the effects of other gangliosides, we made further experiments of Asialo-GM1 and GD1b on the expression of Ubiquilin 1. Western blot showed that Asialo-GM1 and GD1b elevated the expression of Ubiquilin 1 little if any at the concentrations of 1–50 μM. Thus, internal disialosyl galactosyl residue of b-series gangliosides GD1b was indispensable for the activation. Furthermore, these results also demonstrate that the activation of Ubiquilin 1 is independent of sialic acid (Asialo-GM1 has no sialic acid residue and GM1 has one residue while GD1b has two). These results suggest that GM1 induces the expression of Ubiquilin 1 significantly via a yet unidentified mechanism. While combined with the previous reports that significant level of antibodies specific to ganglioside GM1 but not to other gangliosides was found in patients with Alzheimer's disease [4], the specificity of GM1 increasing the expression of Ubiquilin 1 is more interesting. However, further investigations are required to extend this finding to comprehend the association with γ-secretase activity and the production of amyloid peptides.

In summary, our current studies demonstrate that GM1 exposure increases the mRNA and protein expression of Ubiquilin 1 in SH-SY5Y APP695 cells and rat mixed cortical neurons. When combined with the observations of Ubiquilin 1 modulating the γ-secretase in the proteolytic processing of APP and GM1 playing a regulatory role on APP processing pathways, these findings offer a putative candidate for further investigation of GM1 mechanisms in the amyloidogenic processing of AD.

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