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Cytotoxic effects of G_{M1} ganglioside and amyloid β-peptide on mouse embryonic neural stem cells

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ABSTRACT

AD (Alzheimer's disease) is a neurodegenerative disease and the most common form of dementia. One of the pathological hallmarks of AD is the aggregation of extracellular Aβs (amyloid β-peptides) in senile plaques in the brain. The process could be initiated by seeding provided by an interaction between G_{M1} ganglioside and Aβs. Several reports have documented the bifunctional roles of Aβs in NSCs (neural stem cells), but the precise effects of G_{M1} and Aβ on NSCs have not yet been clarified. We evaluated the effect of G_{M1} and Aβ-(1–40) on mouse NECs (neuroepithelial cells), which are known to be rich in NSCs. No change of cell number was detected in NECs cultured in the presence of either G_{M1} or Aβ-(1–40). On the contrary, a decreased number of NECs were cultured in the presence of a combination of G_{M1} and Aβ-(1–40). The exogenously added G_{M1} and Aβ-(1–40) were confirmed to incorporate into NECs. The Ras–MAPK (mitogen-activated protein kinase) pathway, important for cell proliferation, was intact in NECs simultaneously treated with G_{M1} and Aβ-(1–40), but caspase 3 was activated. NECs treated with G_{M1} and Aβ-(1–40) were positive in the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay, an indicator of cell death. It was found that G_{M1} and Aβ-(1–40) interacted in the presence of cholesterol and sphingomyelin, components of cell surface microdomains. The cytotoxic effect was found also in NSCs prepared via neurospheres. These results indicate that Aβ-(1–40) and G_{M1} co-operatively exert a cytotoxic effect on NSCs, likely via incorporation into NEC membranes, where they form a complex for the activation of cell death signalling.

Key words: Alzheimer's disease (AD), amyloid β-peptide (Aβ), apoptosis, G_{M1} ganglioside, glycosphingolipid, neural stem cell.

INTRODUCTION

AD (Alzheimer's disease), the most common form of dementia, is a neurodegenerative disease that manifests clinically as progressive memory loss and cognitive impairment. Pathologically, it is characterized by the appearance of senile plaques and neurofibrillary tangles and loss of neurons in the cerebral cortex (Ariga et al., 2008). Elucidation of the pathogenesis of AD, which is not yet fully understood, is an important subject for the development of therapies for this disease. One of the pathological hallmarks and possible causes of AD is the aggregation and accumulation of extracellular Aβs (amyloid β-peptides) in the brain. Aβs, which are produced from amyloid precursor protein by sequential endoproteolytic cleavages by β-secretase and γ-secretase, are originally monomeric, soluble and non-toxic, but become cytotoxic by aggregation and accumulation. Senile plaques seen in the brains of AD patients are mainly composed of the aggregated and accumulated Aβs. The aggregation of Aβs, a critical step in the onset of AD, has been proposed to be initiated by G_{M1} ganglioside (Hayashi et al., 2004; Yanagisawa, 2007).

Gangliosides, sialic acid-containing glycosphingolipids, are ubiquitously expressed in vertebrate cells and body fluids, and are particularly abundant in the nervous system (Yu et al., 2009). In cells, gangliosides are localized primarily, although not exclusively, on the plasma membrane. Together with cholesterol and sphingomyelin, gangliosides are components of cell surface microdomains (glycosphingolipid-enriched microdomains, caveolae or lipid rafts). These specialized microdomain structures have been suggested to serve as important platforms for modulating cell adhesion and signal transduction. In AD brains, G_{M1} ganglioside, one of the major brain gangliosides, is considered to bind to monomeric Aβs and initiate their

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; bFGF, basic fibroblast growth factor; biotin-Ctxb, biotin-conjugated cholera toxin B subunit; CCD, charge-coupled device; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular-signal-regulated kinase; FITC-Aβ-(1–40), FITC-conjugated Aβ-(1–40); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IACUC, Institutional Animal Care and Use Committee; IL, interleukin; MAP2, microtubule-associated protein 2; MAPK, mitogen-activated protein kinase; N2-DMEM/F12, N2-supplemented DMEM/Ham's Nutrient Mixture F12; NEC, neuroepithelial cell; NSC, neural stem cell; RT–PCR, reverse transcription–PCR; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

aggregation and accumulation by serving the role of 'seeding' (Hayashi et al., 2004; Yanagisawa, 2007). On the other hand, gangliosides are widely known to have neuroprotective effects (Svennerholm, 1994; Ledeen and Wu, 2002; Svennerholm et al., 2002). Certain gangliosides, including G_{M1}, have also been reported to inhibit Aβ-induced secretion of pro-inflammatory cytokines such as IL-1β (interleukin-1β), IL-6 and tumour necrosis factor-α, which are involved in the pathogenic events of AD (Ariga and Yu, 1999; Ariga et al., 2001a). Understanding the relationship between Aβ and G_{M1} and their bidirectional effects is important to fully elucidate the pathogenesis of AD.

At present, there is still no well-established cure for AD. The strategy of using endogenous or transplanted NSCs (neural stem cells) to compensate for the neuronal loss in AD brains has been proposed. Because of their basic biological importance and latent clinical usefulness for treating a variety of neurodegenerative diseases (such as AD), NSCs (which are undifferentiated neural cells that are endowed with a high potential for proliferation and the capacity for self-renewal retaining the multipotency to differentiate into neuronal and glial cells) have been of great interest during the last two decades (Sugaya, 2003). In adult mammalian brains, NSCs are localized in the subventricular zone of the lateral ventricles and the subgranular layer of the dentate gyrus in the hippocampus. Nevertheless, neurogenesis in these areas does not compensate for neuronal loss in AD brains, implying the possibility that this impairment of neurogenesis, especially in the hippocampal dentate gyrus, is involved in the pathogenesis of AD. So far, several reports have documented the bifunctional roles of Aβs in NSCs *in vitro*; Aβs have neurogenic effects in some studies (Lopez-Toledano and Shelanski, 2004; Waldau and Shetty, 2008; Chen and Dong, 2009; Sotthibundhu et al., 2009), but cytotoxic effects in other studies (Haughey et al., 2002a, 2002b; Millet et al., 2005; Calafiore et al., 2006; Waldau and Shetty, 2008). However, the effects of a combination of Aβ and G_{M1} on NSCs have not yet been clarified. In the present study, we evaluated the effects of G_{M1} and Aβ-(1–40), the isoform of 40 amino acid residues in length, on mouse NEC (neuroepithelial cell), which are known to be rich in NSCs (Fukuda et al., 2007).

MATERIALS AND METHODS

Materials

The G_{M1} ganglioside used in the present study was isolated from human brain in our laboratories (Ledeen and Yu, 1982). Aβ-(1–40) was purchased from Bachem Americas (Torrance, CA, U.S.A.; catalogue number H-1194.0001; lot number 2500610). Tunicamycin, an inhibitor of N-linked glycosylation, was purchased from Sigma–Aldrich (St Louis, MO, U.S.A.).

NEC culture

NECs were prepared from telencephalons of mouse embryos (embryonic day 14.5) and cultured in N2-DMEM/F12 [N2-supplemented DMEM (Dulbecco's modified Eagle's medium)/Ham's Nutrient Mixture F12] containing bFGF (basic fibroblast growth factor; Peprotech, Rocky Hill, NJ, U.S.A.) on dishes coated with poly-L-ornithine and fibronectin (Sigma–Aldrich) by the method described by Nakashima et al. (1999). Mice used for the cell preparation were treated according to the guidelines of the IACUC (Institutional Animal Care and Use Committee) of the Medical College of Georgia to minimize pain or discomfort. NECs cultured for 6 days were replated for treatment with G_{M1} and/or Aβ-(1–40). For treatment of NECs, G_{M1} was completely dried under a stream of nitrogen and then dissolved in N2-DMEM/F12 at 37°C. Aβ-(1–40) dissolved in PBS and stored at −80°C as a stock solution was directly added to N2-DMEM/F12 immediately before treatment of NECs; any procedures to induce the formation of the oligomeric or fibrillary form of Aβ-(1–40) were not performed. The NECs treated with G_{M1} and/or Aβ-(1–40) were used for the experiments described below.

WST-8 assay

The number of cells cultured in the presence or absence of G_{M1} and/or Aβ-(1–40) on poly-L-ornithine- and fibronectin-coated 96-well plates for 4 days was estimated by the WST-8 assay, a highly sensitive and reproducible method (Kanemura et al., 2002; Yu and Yanagisawa, 2007; Yanagisawa and Yu, 2009), using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The A_{450} of WST-8-formazan produced by the dehydrogenase activity in the living cells was measured (reference: 650 nm) using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Cell staining

NECs cultured in N2-DMEM/F12 containing G_{M1} and/or Aβ-(1–40) or FITC-Aβ-(1–40) [FITC-conjugated Aβ-(1–40); Bachem Americas] on chamber slides (Nalge Nunc International, Naperville, IL, U.S.A.) for 2 or 3 days were fixed in PBS containing 4% (w/v) paraformaldehyde and stained with biotin-Ctxb (biotin-conjugated cholera toxin B subunit), rat 401 anti-nestin monoclonal antibody (BD Biosciences, San Jose, CA, U.S.A.) or anti-β-III tubulin monoclonal antibody (Sigma–Aldrich). Biotin-Ctxb and monoclonal antibodies were detected with rhodamine-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, U.S.A.) and Alexa Fluor® 488-conjugated anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, U.S.A.) respectively. Nuclei were stained with Hoechst 33258 (Sigma–Aldrich). The stained NECs were photographed under a Nikon Eclipse TE300 fluorescent microscope (Nikon Instruments, Melville, NY, U.S.A.) equipped with a Magnafire digital CCD camera (charge-coupled device camera; Optronics, Goleta, CA, U.S.A.).

Western-blot analysis

Western-blot analysis was performed as previously described (Yanagisawa and Yu, 2009). As primary antibodies, anti-phospho-ERK (extracellular-signal-regulated kinase) monoclonal antibody (Cell Signaling Technology, Danvers, MA, U.S.A.), anti-ERK polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-$p27^{Kip1}$ monoclonal antibody (BD Biosciences), anti-β-actin monoclonal antibody (Sigma–Aldrich) and anti-caspase 3 rabbit monoclonal antibody (Cell Signaling Technology) were used. Horseradish-peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare Life Sciences, Piscataway, NJ, U.S.A.) and anti-rabbit IgG antibody (GE Healthcare Life Sciences) were used as the secondary antibodies. Protein bands reacted with the antibodies were detected using WesternLightning Western Blot Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences, Waltham, MA, U.S.A.).

RT–PCR (reverse transcription–PCR)

RT–PCR was performed as previously described (Ngamukote et al., 2007). The sequences of primer sets were as follows: 5′-TGGAAGTGGCTACATACAGGAC-3′ and 5′-GGTATTAGGCAAGGGGGAAG-3′ for nestin; 5′-CCTCAGCTGACAGAGAAACAG-3′ and 5′-CTTGGTTCTGTGCTCTGTTTTC-3′ for MAP2 (microtubule-associated protein 2); 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling)

Apoptotic cells were detected with the TUNEL assay. In brief, cells were plated on to chamber slides and cultured in N2-DMEM/F12 containing G_{M1} and Aβ-(1–40) in the presence of 5 ng/ml of bFGF for 3 days. Then, the cells were fixed in PBS containing 4% paraformaldehyde for 1 h at room temperature (approx. 25℃) and permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min at 4℃. The cells were incubated with fluorescein-conjugated TUNEL reaction mixture (Roche Diagnostics, Mannheim, Germany) for 2 h at 37℃. Nuclei were stained with Hoechst 33258. Stained cells were photographed under a Nikon Eclipse TE300 fluorescent microscope equipped with a Magnafire digital CCD camera.

G_{M1} binding assay

Physical interaction between G_{M1} and Aβ-(1–40) was revealed by a G_{M1} binding assay using G_{M1}-coated 96-well plates and FITC-Aβ-(1–40). In brief, polystyrene 96-well white plates (Nalge Nunc International) were coated with G_{M1} by adding a methanolic solution of G_{M1} (0, 1 or 10 nmol per well) and/or cholesterol (Sigma–Aldrich; 0 or 7.5 nmol per well) and sphingomyelin (Sigma–Aldrich; 0 or 7.5 nmol per well) to each well; the molar proportion of the lipids was 40:30:30 (G_{M1}/cholesterol/sphingomyelin) (Kakio et al., 2001). After the methanol solvent had been evaporated at 37℃, a blocking solution of PBS containing 1% BSA was added. G_{M1} immobilized on the wells was incubated with Hanks buffered saline solution containing 5 μM of an FITC-Aβ-(1–40) solution in the dark for 1 h at room temperature. After washing, the fluorescent intensity of FITC-Aβ-(1–40) binding to G_{M1} immobilized on the wells was measured using a Victor^3 V multilabel plate reader (PerkinElmer Life and Analytical Sciences) equipped with a λ_{ex}=485 nm filter and a λ_{em}=535 nm filter. To evaluate the efficiency of the assay, the polystyrene 96-well plates coated with G_{M1} (0, 1, 10, 100 and 1000 pmol per well) were incubated with 1 μM of biotin-Ctxb and then 2 μg/ml of Cy2-conjugated streptavidin (Jackson ImmunoResearch).

NSC culture

NSCs were prepared in the form of neurospheres, floating clonal aggregates formed by NSCs *in vitro*, according to previously described methods but with slight modifications (Reynolds et al., 1992; Nakatani et al., 2010). In brief, single-cell suspensions prepared from striata of mouse embryos (embryonic day 14.5) by mechanical trituration were cultured in N2-DMEM/F12 containing 20 ng/ml bFGF and 20 ng/ml epidermal growth factor (Peprotech). Neurospheres formed after 1 week were collected for passage or further analyses. Confirmation of NSCs was performed by cell staining using a subclass control IgG (BD Biosciences) or anti-nestin monoclonal antibody.

RESULTS

First, we examined the number of NECs treated with low concentrations of G_{M1} and Aβ-(1–40). As shown in Figure 1, lower concentrations of G_{M1} (1, 5 or 10 μM) and/or Aβ-(1–40) (1 or 5 μM) have almost no effect on the NEC number. It has been reported that the effect of Aβ on NSCs is highly dependent on the concentration; monomeric Aβ showed no significant effect on proliferation and differentiation of adult mouse NSCs at lower concentrations, but inhibited the proliferation and neurogenesis at higher concentrations (Heo et al., 2007). Therefore we evaluated the effects of higher concentrations of G_{M1} and Aβ-(1–40) by culturing NECs in the presence of 40 μM G_{M1} and 10 μM Aβ-(1–40) on NECs. Neither 40 μM G_{M1} nor 10 μM Aβ-(1–40) affected the NEC number (Figure 2). However, in NECs cultured in the presence of both 40 μM G_{M1} and 10 μM Aβ-(1–40), a significant reduction in the cell number was detected (Figure 2). This result indicates that Aβ-(1–40) in the presence of G_{M1} has a cytotoxic effect on NECs.

Generally, gangliosides exogenously added to tissue culture have been expected to be incorporated into the cell

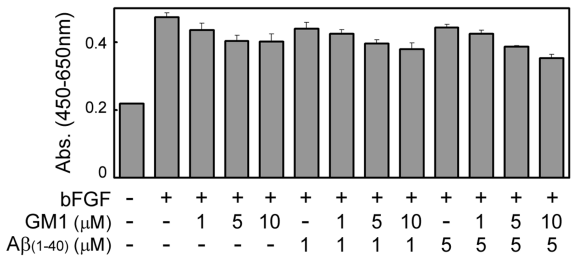

Figure 1 Effects of low concentrations of G_{M1} and Aβ-(1–40) on NECs
The number of NECs cultured in the presence of bFGF (0 or 5 ng/ml), G_{M1} (0, 1, 5 or 10 μM) and Aβ-(1–40) (0, 1 or 5 μM) for 4 days was estimated by the WST-8 assay. bFGF was added as a mitogen of NECs. The absorbance (Abs.) measured at a wavelength of 450 nm (reference: 650 nm) by this assay is highly correlated with the number of living NECs.

membranes through the hydrophobic ceramide residues. However, it is unclear whether exogenously added G_{M1} and Aβ-(1–40) were incorporated into NECs. Taking advantage of barely detectable quantities of G_{M1} expressed in NECs (Yanagisawa et al., 2006a), we evaluated the incorporation of G_{M1} into the NECs by staining with Ctxb, a probe recognizing G_{M1} and other related gangliosides. Simultaneously, Aβ-(1–40) distribution was examined by culturing NECs with FITC-Aβ-(1–40). As shown in Figure 3, G_{M1}-treated NECs showed a strong reactivity to Ctxb. In addition, a strong FITC signal was found in NECs treated with FITC-Aβ-(1–40). These results indicate that exogenously added G_{M1} and Aβ-(1–40) were efficiently incorporated into NECs. In the NECs simultaneously treated with G_{M1} and Aβ-(1–40), however, there was no difference in their fluorescence intensities. Therefore it was confirmed that the co-operative effect of G_{M1} and Aβ-(1–40) on the number of NECs is mediated after the incorporation into NECs.

We then analysed the molecular mechanism underlying the reduction of the NEC number by G_{M1} and Aβ-(1–40). The Ras–MAPK (mitogen-activated protein kinase) pathway is known to be essential for the proliferation of NECs (Yanagisawa et al., 2005). In NECs treated with G_{M1} and/or Aβ-(1–40), however,

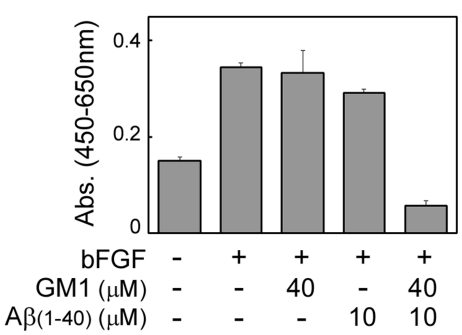

Figure 2 Effects of high concentrations of G_{M1} and Aβ-(1–40) on NECs
The number of NECs cultured in the presence of bFGF (0 or 5 ng/ml), G_{M1} (0 or 40 μM) and Aβ-(1–40) (0 or 10 μM) for four days was estimated by the WST-8 assay. Abs., absorbance.

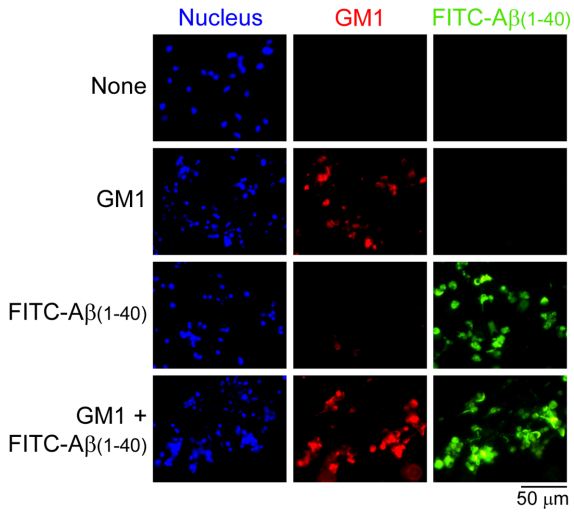

Figure 3 Incorporation of exogenously added G_{M1} and Aβ-(1–40) into NECs
NECs were cultured in the presence of G_{M1} (0 or 40 μM) and FITC-Aβ-(1–40) (0 or 10 μM) for 2 days, and then stained with biotin-Ctxb and rhodamine-conjugated streptavidin. Nuclei were stained with Hoechst 33258.

bFGF-induced activation of the Ras–MAPK pathway was intact (Figure 4A). A cyclin-dependent kinase inhibitor, $p27^{Kip1}$, which has been suggested to be up-regulated by ganglioside stimulation and involved in ganglioside-induced inhibition of neural cell proliferation (Nakatsuji and Miller, 2001), was not up-regulated in NECs treated with G_{M1} and/or Aβ-(1–40) (Figure 4B). These results indicate that G_{M1} and Aβ-(1–40) did not inhibit the proliferation of NECs, at least not at these signalling steps. Generally, the process of differentiation decreases the proliferation rate of stem cells. In fact, it has been reported that a low concentration of Aβ-(1–42) enhances neuronal differentiation of adult NSCs (Heo et al., 2007). In NSCs treated with G_{M1} and/or Aβ-(1–40), however, expression of nestin, a marker protein of NSCs, was not down-regulated (Figures 5A and 5C). The number of cells positive for β-III tubulin, a marker protein of mature neurons scarcely found in untreated NECs, was not increased in G_{M1}- and Aβ-(1–40)-treated NECs (Figure 5B). MAP2, a marker protein of immature and mature neurons, was not up-regulated in G_{M1}- and Aβ-(1–40)-treated NECs (Figure 5C). These results indicate that the NECs treated with G_{M1} and/or Aβ-(1–40) were not differentiated. On the other hand, it has been reported that Aβ-(1–40) and Aβ-(1–42) induce apoptotic cell death in substantia nigra/neuroblastoma cell line cells (Le et al., 1995) and adult NSCs (Heo et al., 2007) respectively. As well, in NECs treated with G_{M1} and/or Aβ-(1–40), caspase 3 (a critical executioner of apoptosis or programmed cell death signalling) was synergistically activated (Figure 6A). It was confirmed that a number of NECs treated with G_{M1} and Aβ-(1–40) were positive for the TUNEL assay, an indicator of cell death accompanied by DNA fragmentation (Figures 6B and 6C). These results suggest that the reduction of NEC number by G_{M1} and Aβ-(1–40) was

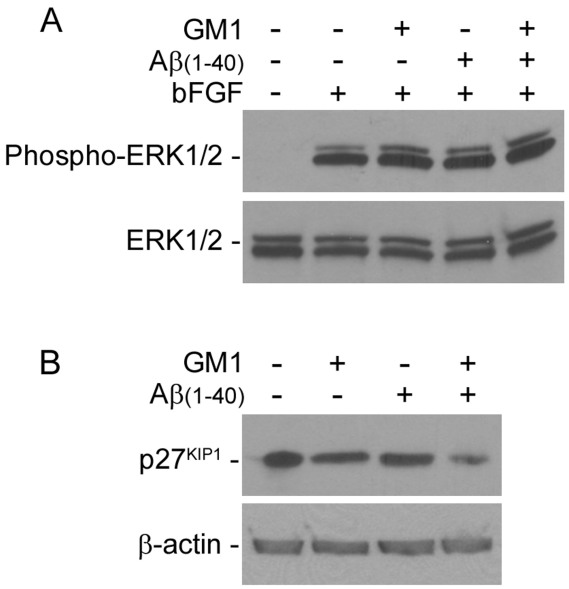

Figure 4 Cell proliferation signalling in NECs treated with G_{M1} and Aβ-(1–40)

(**A**) Activation of ERK (MAPK) in NECs treated with G_{M1} (0 or 40 μM) and Aβ-(1–40) (0 or 10 μM) for 2 days and then stimulated with bFGF (0 or 10 ng/ml) for 10 min was analysed by Western blotting. bFGF was used as an inducer of ERK activation. (**B**) Expression of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor up-regulated by ganglioside stimulation and involved in ganglioside-induced inhibition of neural cell proliferation, in NECs treated with G_{M1} (0 or 40 μM) and Aβ-(1–40) (0 or 10 μM) for 2 days was analysed by Western blotting.

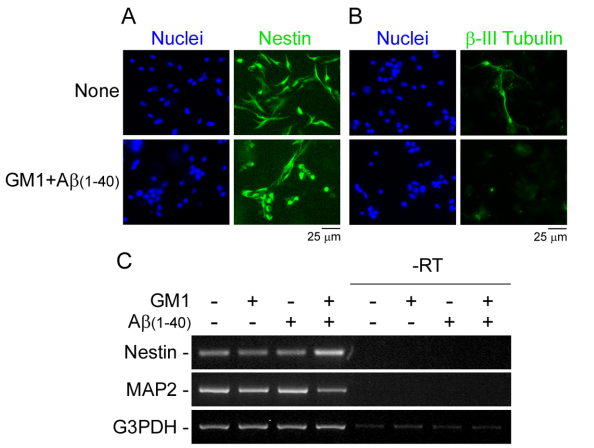

Figure 5 Neural lineage marker expression in NECs treated with G_{M1} and Aβ-(1–40)

Expression of (**A**) nestin (a marker protein of neural stem cells) and (**B**) β-III tubulin (a marker protein of mature neurons) in NECs treated with G_{M1} (0 or 40 μM) and Aβ-(1–40) (0 or 10 μM) for 3 days was analysed by cell staining. Nuclei were stained with Hoechst 33258. (**C**) Expression of nestin and MAP2 (a marker gene of immature and mature neurons) in NECs treated with G_{M1} (0 or 40 μM) and Aβ-(1–40) (0 or 10 μM) for 2 days was analysed by RT-PCR. 'G3PDH' indicates control GAPDH. '−RT' indicates negative controls without reverse transcription.

caused, at least in part, by activation of the cell death process, but not by the inhibition of proliferation or the induction of differentiation, after incorporation into the NECs.

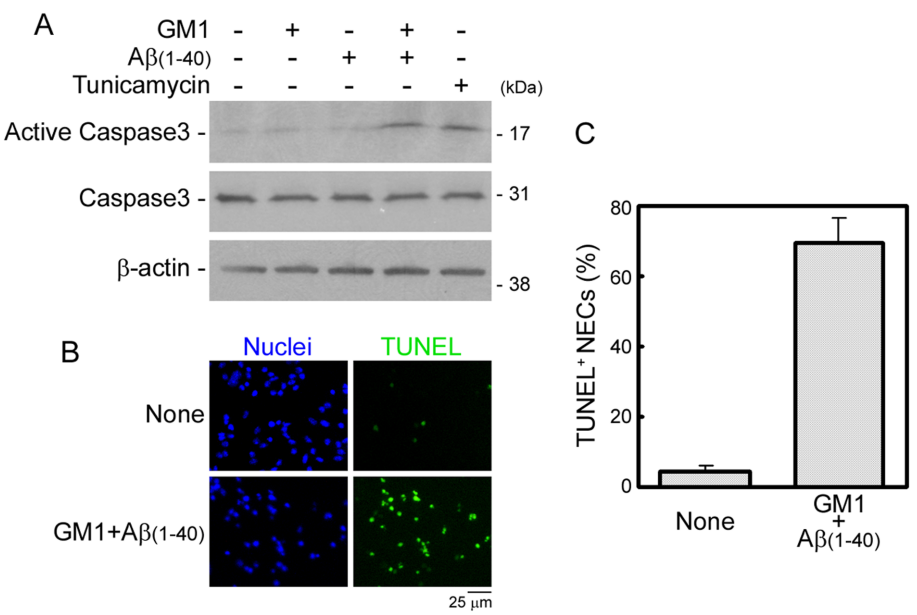

Figure 6 Apoptosis of NECs treated with G_{M1} and Aβ-(1–40)

(**A**) Activation of caspase 3 (a critical executioner of apoptosis or programmed cell death signalling) in NECs treated with G_{M1} (0 or 40 μM) and Aβ-(1–40) (0 or 10 μM) in the presence of bFGF (5 ng/ml) for 2 days was analysed by Western blotting. An inhibitor of N-linked glycosylation, tunicamycin (1 μg/ml for 10 h), was used as a positive control to activate stress-mediated cell death signalling. (**B**) Apoptotic cells in NECs treated with G_{M1} (0 or 40 μM) and Aβ-(1–40) (0 or 10 μM) in the presence of bFGF (5 ng/ml) for 3 days were detected with the TUNEL assay. Nuclei were stained with Hoechst 33258. (**C**) The proportion of TUNEL-positive cells in NECs treated with or without G_{M1} and Aβ-(1–40).

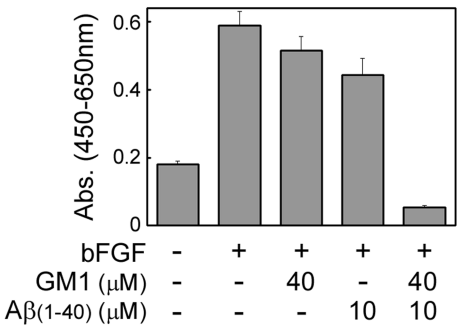

Figure 7 Effects of Aβ-(1–40) pre-incubated with G_{M1} on NECs
To evaluate whether the cytotoxic effect of Aβ-(1–40) and G_{M1} can be inhibited by sequestering, Aβ-(1–40) (0 or 10 μM) was pre-incubated with G_{M1} (0 or 40 μM) at 37°C for 30 min to allow their binding. The number of NECs cultured in N2-DMEM/F12 containing the Aβ-(1–40) pre-incubated with G_{M1} in the presence of bFGF (0 or 5 ng/ml) for 4 days was estimated by the WST-8 assay. Abs., absorbance.

Our previous study using surface plasmon resonance and a liposome capture method showed that G_{M1} directly bound to Aβ with high affinity (Ariga et al., 2001b). This finding suggests that there is a possibility that sequestering Aβ-(1–40) with G_{M1} can inhibit the cytotoxic effect. To evaluate this possibility, we pre-incubated Aβ-(1–40) with G_{M1} to allow their binding and then cultured NECs in the presence of this pre-incubated mixture. However, the cytotoxic effect of Aβ-(1–40) was not inhibited by pre-incubation with G_{M1}; 10 μM of Aβ-(1–40) pre-incubated with 40 μM of G_{M1} drastically reduced the NEC number (Figure 7). Thus we confirmed the physical interaction between G_{M1} and Aβ-(1–40) by the G_{M1} binding assay. First, we confirmed the efficiency of the assay by detecting the interaction between G_{M1} and the well-known ligand, Ctxb. As shown in Figure 8(A), this assay could dose-dependently detect binding of Ctxb to G_{M1} immobilized on 96-well plates. Then, we analysed the physical interaction between G_{M1} and FITC-Aβ-(1–40) using this assay. As shown in Figure 8(B), FITC-Aβ-(1–40) strongly interacts with G_{M1} only in the presence of cholesterol and sphingomyelin, components of cell surface microdomains, as reported by Kakio et al. (2001); the interaction between G_{M1} and FITC-Aβ-(1–40) was relatively weak in the absence of cholesterol and sphingomyelin. This result suggests that G_{M1} itself could not sequester Aβ-(1–40) *in vitro* and reduce the cytotoxicity. So far, the existence of cell surface microdomains, which are rich in cholesterol and sphingomyelin, has been confirmed in NECs (Yanagisawa et al., 2004). It is expected that exogenously added G_{M1} is incorporated into NECs and distributed to the cell surface microdomains. Binding of Aβ-(1–40) to NECs and activation of cell death signalling may occur in G_{M1}-positive microdomains.

As we have described, NECs are known to be rich in NSCs. However, NECs are still a heterogeneous cell population containing a few differentiated cells. To confirm the cytotoxic effect of G_{M1} and Aβ-(1–40) on NSCs, we prepared NSCs from striata of mouse embryos in the form of neurospheres

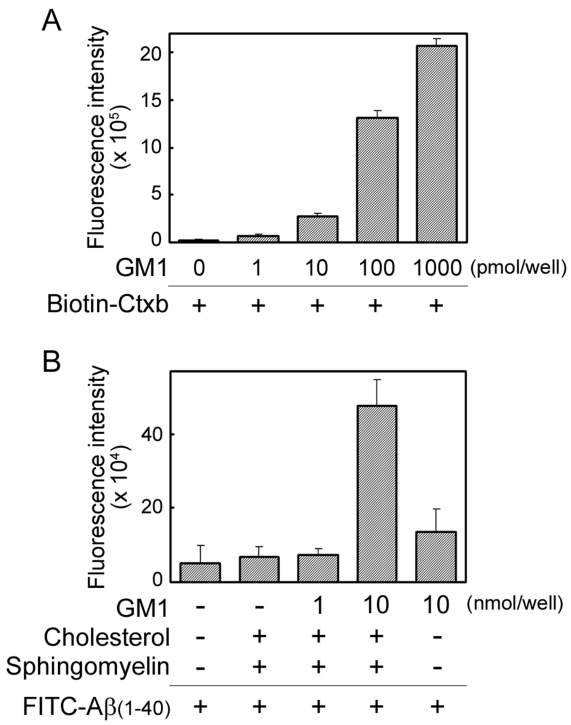

Figure 8 Physical interaction of G_{M1} and Ctxb (A) or Aβ-(1–40) (B)
(**A**) G_{M1} (0, 1, 10, 100 or 1000 pmol per well) on polystyrene 96-well white plates was incubated with 1 μM of biotin-Ctxb and then 2 μg/ml of Cy2-conjugated streptavidin. (**B**) G_{M1} (0, 1 or 10 nmol per well), cholesterol (0 or 7.5 nmol per well) and sphingomyelin (0 or 7.5 nmol per well) on polystyrene 96-well white plates were incubated with 5 μM of FITC-Aβ-(1–40). The fluorescence intensities, which reflect the physical interaction of G_{M1} with Ctxb or FITC-Aβ-(1–40), were measured using a Victor^3 V multilabel plate reader equipped with a λ_{ex}=485 nm filter and a λ_{em}=535 nm filter.

(Figure 9A). These NSCs were confirmed to be positive for the marker protein, nestin (Figure 9B). A number of G_{M1}- and Aβ-(1–40)-treated NSCs were found to be positive in the TUNEL assay (Figures 9C and 9D). These results indicate that G_{M1} and 10 μM of Aβ-(1–40) also have a cytotoxic effect on NSCs.

DISCUSSION

In the present study, we clearly showed that G_{M1} and Aβ-(1–40) co-operatively have cytotoxic effects on NECs. This finding suggests the possibility that a combination of Aβ and G_{M1} is cytotoxic to not only neurons, but also NSCs in AD brains. In adult mammalian brains, G_{M1} is well known to be highly expressed (Ngamukote et al., 2007; Yu et al., 2009). The expression of G_{M1} may be involved in the impairment of neurogenesis in the AD brains. It has been reported that neural precursor cells isolated from post-mortem AD patient brains exhibit a severe reduction in number during culture (Lovell et al., 2006). Aβ and G_{M1} may be involved in this reduction in the

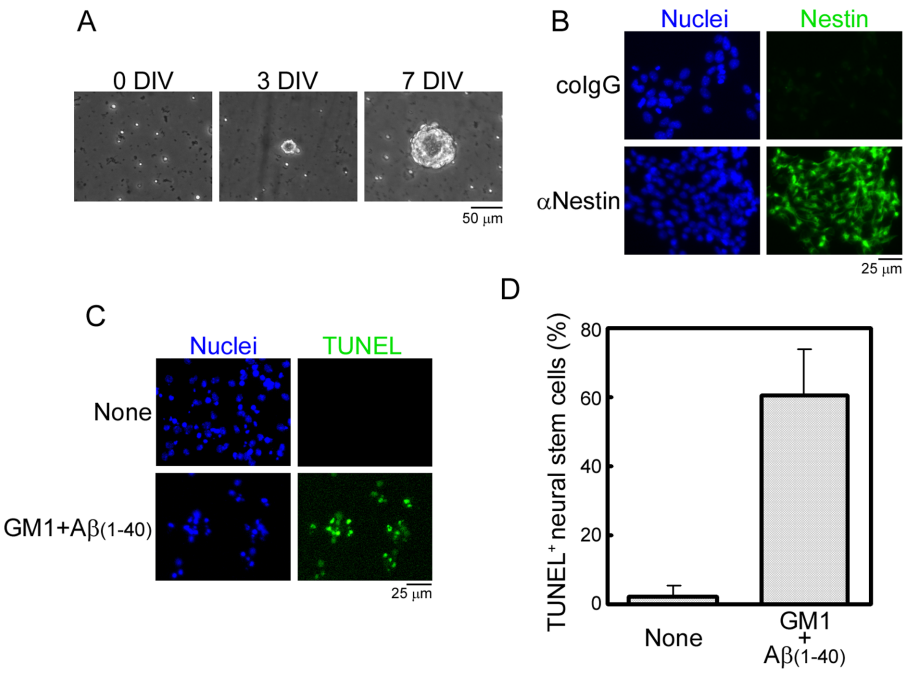

Figure 9 **Effects of G_{M1} and Aβ-(1–40) on NSCs**

(**A**) NSCs prepared from striata of mouse embryos in the form of neurospheres at 0, 3 and 7 days *in vitro* (DIV). (**B**) NSCs stained with subclass control IgG (colgG) or anti-nestin antibody. Nuclei were stained with Hoechst 33258. (**C**) Apoptotic cells in NSCs treated with G_{M1} (0 or 40 μM) and Aβ-(1–40) (0 or 10 μM) in the presence of bFGF (5 ng/ml) for 3 days were detected with the TUNEL assay. Nuclei were stained with Hoechst 33258. (**D**) The proportion of TUNEL-positive cells in NSCs treated with or without G_{M1} and Aβ-(1–40).

number of neural precursor cells. In pathological conditions, however, the involvement of gangliosides other than G_{M1} should also be considered. For instance, Aβs have been reported to bind to rat phaeochromocytoma, PC12 cells; the binding does not seem to be with G_{M1}, but rather with fucosylated G_{M1}, which is expressed (Yanagisawa et al., 2006b). In addition, an *in vitro* study using surface plasmon resonance clarified that α-series gangliosides ($G_{Q1b\alpha}$ and $G_{T1a\alpha}$) and b-series gangliosides (G_{Q1b}, G_{T1b}, G_{D3} and G_{D1b}) and G_{D1a} have higher affinities for Aβ than G_{M1} (Ariga et al., 2001b). More importantly, a recent analysis of G_{D3} synthase-knockout mice cross-bred with AD model mice has suggested that the *bona fide* gangliosides initiating Aβ aggregation in the AD brains are b-series gangliosides, but not G_{M1} (Bernardo et al., 2009). Therefore other gangliosides expressed in NECs such as G_{D3}, $G_{Q1b\alpha}$ and $G_{T1a\alpha}$ (Yanagisawa et al., 2004; Ngamukote et al., 2007) may also increase the neurotoxic effects of Aβ on NSCs in the pathological condition of AD. Although there have been many reports so far, the functional roles of Aβs on NSCs are still controversial, perhaps because of the ganglioside species differentially expressed in these cells. Evaluation of the effects of exogenous G_{M1} on neurogenesis and pathogenesis of AD under pathological conditions, for instance using AD model mice (Jankowsky et al., 2001), will be an interesting and fruitful subject for future studies. These studies to understand the roles of G_{M1} and Aβ on NSCs in AD may contribute to the development of new regenerative therapies for this disease.

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