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Effects of Amyloid β -Peptides and Gangliosides on Mouse Neural Stem Cells

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Abstract

The interaction of amyloid β -proteins (A β s) with membrane lipids has been postulated as an early event in A β fibril formation in Alzheimer's disease. We evaluated the effects of several putative bioactive A β s and gangliosides on neural stem cells (NSCs) isolated from embryonic mouse brains or the subventricular zone of adult mouse brains. Incubation of the isolated NSCs with soluble A β 1–40 alone did not cause any change in the number of NSCs, but soluble A β 1–42 increased their number. Aggregated A β 1–40 and A β 1–42 increased the number of NSCs but soluble and aggregated A β 25–35 decreased the number. Soluble A β 1–40 and A β 1–42 did not affect the number of apoptotic cells but aggregated A β 1–40 and A β 1–42 did. When NSCs were treated with a combination of GM1 or GD3 and soluble A β 1–42, cell proliferation was enhanced, indicating that both GM1 and GD3 as well as A β s are involved in promoting cell proliferation and survival of NSCs. These observations suggest the potential of beneficial effects of using gangliosides and A β s for promoting NSC proliferation.

Keywords

Alzheimer's disease; Amyloid β -peptide; Ganglioside; Neural stem cell Cell; proliferation

Introduction

Alzheimer's disease (AD) is the most common form of dementia with clinical symptoms that include deficits in memory, judgment, thinking, and behavior. Those symptoms usually develop slowly and become worse over time, interfere with daily tasks, and ultimately lead to death. It is well accepted that deposition of aggregated amyloid β -protein (A β) to form amyloid plaques, also known as senile plaques (SPs), together with associated reactive astrocytosis and dystrophic neuritis, represent major pathological hallmarks of AD [1]. During neurodegeneration, the physicochemical properties of membranes are altered, which

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results in imbalances in the proportion of membranous lipids. Among the various lipid changes, the most notable are observed with gangliosides during the progression of AD [2].

Gangliosides are sialic acid-containing glycosphingolipids expressed primarily in the outer leaflet of the plasma membrane of all vertebrate cells and are particularly abundant in the nervous system [3, 4]. The expression of neuronal gangliosides changes dramatically during cellular differentiation and brain development. For instance, in rodent brains a shift from the synthesis of simple gangliosides, such as GM3 and GD3, to the synthesis of the more complex gangliosides in the a- and b-series during brain development has been well documented [5, 6]. Remarkably, GD3 is predominant in embryonic brain, particularly in NSCs, and accounts for over 80 % of the total ganglioside species in those cells. On the other hand, the level of GM1 increases in mature brain and differentiated neural cells. Several reports have indicated that gangliosides can interact with A β s with high affinities [7, 8], and that this interaction may result in conformational changes of A β s [8–10]. Histochemical analysis of amyloid plaques in the SPs of AD brains revealed the presence of the GM1-bound A β s, which may act as an endogenous seed for A β fibrillogenesis [11].

Unfortunately, at present there has not been any effective therapy to halt the progression of AD. Cognitive enhancers such as donepezil and memantine have been used [12], but these drugs can only help to ameliorate the symptoms. With the advantage of cell replacement therapy, it is expected that the use of neural stem cells (NSCs) may contribute to the treatment of AD as well as several other neurodegenerative disorders. NSCs are undifferentiated neural cells characterized by their high proliferative potential and the capacity for self-renewal with retention of multipotency, i.e., generating brain-forming cells such as neurons, astrocytes, and oligodendrocytes. For the therapeutic use of NSCs in AD, detailed clarification of the effects of A β s and gangliosides on NSCs seems to be warranted. In this regard, it is worth noting that A β s have been reported to possess neurogenic effects on NSCs [13–17]. Nevertheless, the effects of A β s on NSCs are still controversial because A β s have also been reported to be cytotoxic in some other studies [18–21]. This discrepancy may result from the difference of the patterns and levels of gangliosides expressed in the NSCs. In fact, we have recently reported that a combination of A β 1–40 and GM1 at relatively high concentrations (submillimolar) co-operatively exerted a cytotoxic effect on mouse embryonic NSCs [22]. Furthermore, the effects of other forms of A β s (e.g., A β 25–35 and A β 1–42) and gangliosides on NSCs have never been evaluated. In addition, the effects of the aggregated forms of A β s, which are known to be present in physiological conditions, with gangliosides on NSCs also have not been investigated. In this research, we carried out a systematic study to investigate the effects of various forms of A β s (A β 25–35, A β 1–40, and A β 1–42) and several representative gangliosides (GM1, GD3, and GM3) on mouse NSCs isolated from embryonic brains and the subventricular zone (SVZ) of adult brains.

Materials and Methods

Materials

GM1, GD3 and GM3 used in this study were isolated from either human brains or bovine buttermilk in our laboratory by established procedures [23–25]. A β 1–40, A β 1–42 and A β 25–

35 were purchased from Bachem Americas (Torrance, CA). To prepare the aggregated forms of A β s, the peptide solutions were incubated at 37 °C for 3 days before use [13, 14].

Neural Stem Cell Culture

Mouse NSCs were prepared from embryonic brains or SVZ of adult brains in the form of neurospheres, which are floating clonal aggregates formed by NSCs in vitro [26]. In brief, single-cell suspensions prepared from the striata of embryonic mouse brains at E14.5 or the SVZ of adult mouse brains by trypsinization and mechanical trituration were cultured in Neurobasal A medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen), 20 ng/ml of basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ), and 20 ng/ml of epidermal growth factor (EGF) (Peprotech). Neurospheres formed after 1 week were collected for passage or further analyses. The use of animals for this study was approved by the Institutional Animal Care and Use Committees at Georgia Regents University and Charlie Norwood Veterans Affairs Medical Center, Augusta, GA.

WST-8 Assay

The number of NSCs cultured in the presence or absence of gangliosides and/or A β s was estimated by the WST-8 assay using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). For neurosphere experiments (Fig. 1), NSCs dissociated from neurospheres were plated onto non-coated 96-well plates at a density of 1×10^4 cells per well. At the time the culture was started, gangliosides and A β were dissolved in media. For monolayer culture of NSCs (Figs. 2, 3, 4, and 5), the dissociated NSCs from neurospheres were plated onto 96-well plates that had been coated with poly-L-ornithine (Sigma-Aldrich, St. Louis, MO) and fibronectin (Sigma-Aldrich). On the next day, gangliosides and/or A β dissolved in media were added to each well. The total volume of culture media was 100 μ l/well. After 3 days of culture, a 10- μ l WST-8 solution was added to each well. After incubating 3 h in a CO₂ incubator, the absorbance of WST-8-formazan produced by the dehydrogenase activity in living neural cells was measured at a wavelength of 450 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The absorbance measured by this assay was highly correlative with the number of living NSCs.

TUNEL Assay

Apoptotic cells were detected with the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay as previously described [22, 26]. In brief, cells were plated onto chamber slides coated with poly-L-ornithine and fibronectin and cultured for 3 days in Neurobasal A medium containing A β s in the presence of 20 ng/ml of bFGF and 20 ng/ml of EGF. The cells were then fixed in PBS containing 4 % paraformaldehyde for 1 h at room temperature and permeabilized in 0.1 % sodium citrate containing 0.1 % Triton X-100 for 2 min at 4 °C. The cells were incubated with the fluorescein-conjugated TUNEL reaction mixture (Roche Diagnostics, Mannheim, Germany) for 2 h at 37 °C. Nuclei were stained with Hoechst 33258. The stained cells were photographed under a Nikon Eclipse TE300 fluorescent microscope equipped with a digital CCD camera.

Statistical Evaluation

Data are expressed as mean $\pm$ standard error of the mean (SEM) from three to nine independent experiments. Statistical significance was determined using unpaired two-tailed Student's *t* test, and $p < 0.05$ was regarded as significant.

Results

Effects of High Concentrations of Gangliosides on Neurospheres

Neurospheres were generated from dissociated cells from the striata of E14.5 embryos or SVZ of adult mice. To measure the cell numbers, the isolated cells from neurospheres were treated with gangliosides, and living cells were counted 3 days after culture. The relative absorbance of the WST-8 assay revealed the percentage of absorbance against vehicle treatment. Both GM1 and GD3 at higher concentrations (50 and 200 μ M) significantly decreased the cell numbers in a dose-dependent manner (Fig. 1b). Smaller neurospheres were observed (data not shown) after treatment with high concentrations of gangliosides. These results indicate that gangliosides GM1 and GD3 can exert an inhibitory effect on cell proliferation on neurospheres in a dose-dependent manner.

Effects of A β s on Neurospheres

Next, the cell numbers were counted after treatments with A β s on neurosphere preparations. Treatment with a soluble form of A β 1–42 (10 μ M) significantly increased the number of cells of neurospheres prepared from both embryonic (116 ± 5 %) and adult (109 ± 3 %) brains (data not shown). Soluble A β 1–40 did not have any effect on the numbers of cells on neurospheres (data not shown).

On the other hand, the aggregated forms of A β 1–40 and A β 1–42 significantly increased the numbers of cells from neurospheres in a dose-dependent manner (Fig. 1c). At high concentrations of A β 1–40 and A β 1–42 (50 μ M), the numbers of cells were elevated to 235 ± 10 % for A β 1–40 and 170 ± 5 % for A β 1–42 of the normal growth rate of embryonic neurospheres, respectively. In the presence of aggregated forms of A β s, the neurospheres appeared larger (data not shown). Increases in the cell number were also found for adult neurospheres treated with the aggregated forms of A β 1–40 and A β 1–42 (data not shown). Under these conditions, the aggregated form of A β 25–35 exhibited inhibitory effects on the number of cells of neurospheres in a dose-dependent manner (Fig. 1c).

Effects of High Concentration of Gangliosides and the Aggregated Forms Of A β s on Neurospheres

The aggregated forms of A β 1–40 and A β 1–42 exhibited proliferative effects of the cells for embryonic and adult neurospheres, as shown in Fig. 1c. Further, we evaluated the additive effects of gangliosides (10, 50, and 100 μ M) and aggregated A β s (10 μ M of A β 1–40 or A β 1–42) on neurospheres. The addition of GM1 or GD3 gangliosides (50 or 100 μ M) inhibited cell growth, with GD3 having greater effects than GM1 in a dose-dependent manner (data not shown).

Effects of A β s and Low Concentrations of Gangliosides on Embryonic NSCs

Recently, we reported that exogenously added A β 1–40 (10 μ M) was incorporated into embryonic NSCs (monolayer culture), and the combination treatment with A β 1–40 (10 μ M) and GM1 at 40 μ M exerted cytotoxic effects on embryonic NSCs [22]. It is unclear, however, whether a lower concentration of gangliosides and A β s had similar effects on embryonic NSCs. To clarify this issue, we first examined the number of embryonic NSCs in monolayer culture after treatment with lower concentrations (1 nM to 10 μ M) of gangliosides. GM1 or GM3 at lower concentrations (1 nM to 1 μ M) significantly promoted cell proliferation for embryonic NSCs (Fig. 2), but GD3 did not (data not shown). Next, we evaluated the combinatorial effects of A β s and low concentrations of gangliosides on cell proliferation of embryonic NSCs. Treatment with soluble A β 1–40 and GD3 (10 nM to 10 μ M) had inhibitory effects for embryonic NSC proliferation (Fig. 3a), but GM1 and GM3 had no effects (data not shown). Soluble A β 1–42 (10 μ M) alone exerted a dramatic stimulatory effect on cell proliferation of embryonic NSC monolayer culture and the number of NSCs increased dramatically (149 ± 10 %) (Fig. 3b). Figure 3b also shows that addition of GM1 and GD3 enhanced proliferative effects of soluble A β 1–42 on embryonic NSCs, whereas GM3 had no additional effects (data not shown). Similar to neurosphere preparations, the numbers of embryonic NSCs grown as monolayer cultures also increased after treatment with aggregated A β 1–40 or A β 1–42 (Figs. 1c, 4). As shown in Fig. 4b, treatment of cells with 100 nM of GM1 significantly increased the cell number on embryonic NSCs treated with aggregated A β 1–42. GM3, however, exerted inhibitory effects on aggregated A β -stimulated cell proliferation (Fig. 4a, b). Similar to neurospheres (Fig. 1c), the number of cells was slightly decreased in monolayer culture of embryonic NSCs treated with both soluble and aggregated forms of A β 25–35 (data not shown).

Apoptosis of NSCs Treated with A β s and Gangliosides

Figure 5 shows the percentage of apoptotic embryonic NSCs after treatment with soluble and aggregated forms of A β s (1–40 and 1–42). No difference was found in the number of cells between NSCs treated with and without soluble forms of A β s. This result was consistent with our previous report that expression of active caspase-3 had no difference between intact cells and NSCs treated with soluble form of A β 1–40 [22]. Taken together, our data suggest that the increase of cell number with soluble A β 1–42 treatment may cause cell proliferation rather than protection from cell death.

In contrast, the number of NSCs undergoing apoptosis decreased significantly by treatment with the aggregated forms of A β s (1–40 and 1–42). Those results indicate that the aggregated forms of A β s, but not the soluble forms, may be involved in the protection of NSCs from undergoing apoptosis. High concentrations of gangliosides (50 μ M of GM1 or GD3) and soluble forms of A β s increased the number of TUNEL⁺ apoptotic cells in embryonic NSC preparations (data not shown). Those results are also consistent with our previous observations [22] that the combined treatment with high concentrations of gangliosides and A β s exhibited enhanced apoptotic effects.

Discussion

Although A β s at elevated concentrations in brain are generally considered to be cytotoxic effect owing to their ability to form fibrils, there has been a growing awareness of the potential beneficial effects on neurogenesis of A β s at low concentrations. Those observations are, however, controversial as some reports also indicate that they are detrimental to neurogenesis. Some of the discrepancies could be traced to the use of different forms and concentrations of A β s, either in the presence or absence of gangliosides that bind with those peptides. For this reason, we first undertook this study with the goal of clarifying the effect of various forms of A β s on NSCs in different conditions using cell proliferation as a criterion. We found that soluble A β 1–42 and aggregated A β s (A β 1–40 and A β 1–42) stimulated cell proliferation in NSCs. This is consistent with several reports demonstrating the stimulatory effects of A β s on the proliferation of NSCs [13, 14, 16, 27]. Most remarkably, several *in vivo* studies also showed that intracerebroventricular infusion of A β 1–42 in nanomolar concentrations promotes neurogenesis in adult mouse brains [18], and A β accumulation increases neurogenesis in the hippocampus and other brain regions in AD transgenic mouse models [28–33]. It is interesting to note that AD patient's brain revealed increased neuronal precursor markers, such as doublecortin and polysialylated neural cell adhesion molecule (PSA-NCAM) in the hippocampus, presumably in response to the shedding of A β s [31]. Our current study confirmed that both soluble and aggregated forms of A β 1–42 have proliferative effects on NSCs.

A β 1–40 and A β 1–42 are two major forms of A β (Fig. 1a), and during normal metabolism of amyloid precursor protein (APP), A β 1–40 is predominantly produced. The A β 1–42 species is usually a minor product, but is more cytotoxic and forms aggregates more easily than A β 1–40 [34]. A β 1–42 is the major peptide constituent of amyloid plaques, and increased production of the 42-amino acid peptide correlates with earlier onset of AD. Approximately 1 % of freshly dissolved A β 1–40 forms dimers [35]. However under the same conditions, about 7.4 % of A β 1–42 is converted to the dimeric form. Thus, it suggests that the soluble A β 1–42 has a higher tendency to exist as dimers. A β 1–42 has less configurational entropy and has been found to confer higher rigidity on the C terminus, which may explain the higher amyloidogenic ability [36]. Yang and Teplow [37] reported that the two additional hydrophobic residues (isoleucine and alanine) in A β 1–42 contribute significantly to the contact between the C terminus and the central hydrophobic clusters. Thereby, the β -sheet structure of A β 1–42 is more stable than A β 1–40. Those structural distinctions of between A β 1–40 and A β 1–42 might affect their biological activities such as their ability to contribute to NSC proliferation. The higher rigidity, less entropy and dimeric stability of A β 1–42 might increase its interaction with NSC membranes, which in turn, modulate its effect on cellular proliferation.

Significant changes in ganglioside patterns in AD brains have been reported. In AD patients brain, major gangliosides such as GM1, GD1a, GD1b and GT1b are all decreased [38–42] and b-series gangliosides such as GD1b and GT1b are preferentially affected. Additionally, in frontal and parietal cortex simple gangliosides, including GM2, GM3, GM4 and GD3, are elevated. These findings suggest that abnormal ganglioside metabolism coincides with the affected brain region of neurodegeneration in AD patients. However, we and others have not

detected significant changes in the major brain ganglioside pattern in the AD mouse brains [43–45] except an increase of minor gangliosides such as GM2 and GM3 in the cerebral cortex [46]. Most interestingly, there is an increase of cholinergic neuronal marker gangliosides such as GT1ac and GQ1ba [43]. Since A β s have a tendency to bind gangliosides, we also examined the ability of gangliosides and combinations of gangliosides and A β s on cell proliferation. In this study, we found that lower concentrations of GM1 and GM3 promote cell proliferation on NSCs (Fig. 2). Other investigators also reported that exogenously added gangliosides exhibited stimulatory effects on cellular proliferation of induced pluripotent stem [47] and vascular smooth muscle cells [48]. We have reported NSCs express GD3 as a predominant ganglioside [26,49, 50] and it is co-localized with EGFR to regulate cellular proliferation induced by EGF (unpublished data). Quite contrarily, it has been reported that GD3 induces mitochondrial permeability transition and this event precedes apoptosis [51], and GD3 is required for Fas-induced cell death and A β -induced cell death [52, 53]. As mentioned in the Result section, treatment of NSCs with a high concentration of GD3 (50 μ M) induced NSCs to undergo apoptosis (>50 %). Thus, the positive or negative effects of gangliosides might depend on culture conditions and ganglioside concentrations. Our results, together with those from other investigators, suggest that gangliosides modulate NSC proliferation or apoptosis depending on the concentrations of gangliosides and the culture conditions of the cells.

With respect to A β 25–35, it does not occur naturally but has been shown to mimic the adverse effects of A β 1–42 in several studies [14]. Interestingly, hippocampal neuronal cultures treated with A β 25–35 showed enhanced metabolism of lipids such as phospholipids (+52 %) and gangliosides (+193 %) [54]. In addition, exposure of rat cultured cortical neurons to A β 25–35 induced a substantial increase of the intracellular GD3 levels [52]. Those reports suggest that A β s can modulate ganglioside metabolism in the NSCs and prompted us to propose that a combined use of A β s and gangliosides in suitable concentrations should promote NSC proliferation. We evaluated the number of embryonic NSCs treated with low concentrations of GM1 and GD3 in the presence of soluble forms of A β s. In embryonic NSC culture, lower concentrations of GD3, combined with soluble A β 1–40, had an inhibitory effect on cellular proliferation. On the other hand, lower concentrations of GM1 and GM3 with A β 1–42 enhanced proliferation of embryonic NSCs. GM1 also promoted cell proliferation with the aggregated form of A β 1–42. These results indicate that appropriate concentrations of both gangliosides and A β s are critically involved in stimulating NSC proliferation.

A β s is produced by cultured cells during normal cellular metabolism [55, 56]. Because A β 1–40 and A β 1–42 are present in the brain and cerebrospinal fluid of normal individuals, it suggests that these peptides are likely physiologically active in normal life [57]. Despite extensive efforts for studying A β s for their cytotoxic effects in AD, the normal biological functions and positive effects of A β s have remained elusive. Gangliosides are known to interact with A β s with high affinities and are known to be involved in conformational changes of A β s. In this study, we evaluated the effect of gangliosides, GM1, GD3 and GM3, and A β s (A β 1–40, A β 1–42, and A β 25–35) on cell proliferation of the NSCs. Aggregated A β 1–40 and soluble or aggregated A β 1–42 were involved in cell proliferation of the NSCs. The lower concentrations of gangliosides and A β s 1–42 induced cell proliferation of NSCs,

suggesting that understanding the roles of gangliosides and A β s on NSCs may help devise new strategies for treatment of AD.

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Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid β -protein
SPs	Senile plaques
NSCs	Neural stem cells
SVZ	Subventricular zone
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

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Fig. 1.

Effects of gangliosides or A β s on cellular proliferation of neurospheres. The number of cells in the neurospheres treated with gangliosides and A β s was estimated by the WST-8 assay. The absorbance measured at a wavelength of 450 nm (reference: 650 nm) in this assay is highly correlated to the number of NSCs. The y-axis represents relative absorbance (Abs.). The relative absorbance of the WST-8 assay revealed the percentage of absorbance against vehicle treatment. **a** Primary structures of A β peptides. **b** Dissociated embryonic neurospheres were cultured for 3 days in the presence of different concentrations (10, 50, or 200 μ M) of GM1 or GD3. **c** Dissociated neurospheres were cultured for 3 days in the presence of different concentrations (1, 10, or 50 μ M) of aggregated A β 1-40, A β 1-42, or A β 25-35. Each *bar* represents mean $\pm$ SEM of 3 independent experiments ($n = 3$). Comparison was made between vehicle versus each treatment. * $p < 0.05$

Fig. 2. Effects of low concentrations of gangliosides on embryonic NSCs. The number of NSCs in monolayer cultures in the presence of GM1 or GM3 ganglioside at 0.001, 0.01, 0.1, 1 or 10 μ M was estimated by the WST-8 assay. The relative absorbance of the WST-8 assay revealed the percentage of absorbance against vehicle treatment. The *bar* denotes mean $\pm$ SEM of 8 independent experiments ($n = 8$). Comparison was made between vehicle versus each treatment. * $p < 0.05$

Fig. 3.

Effects of low concentrations of gangliosides and soluble A β s (A β 1-40 or A β 1-42) on embryonic NSCs in culture. The number of NSCs on monolayer cultures in the presence of gangliosides (0, 0.001, 0.01, 0.1, 1 or 10 μ M of GM1 or GD3) and soluble A β s (10 μ M) was estimated by the WST-8 assay. **a** A β 1-40; **b** A β 1-42. The relative absorbance of the WST-8 assay revealed the percentage of absorbance against vehicle treatment. Each bar denotes mean $\pm$ SEM of 8 independent experiments (n = 8). * p < 0.05: Comparison was made between vehicle versus each treatment. ** p < 0.05: Comparison was made between A β only (0 μ M of ganglioside, white column) versus each treatment

Fig. 4.

Effects of low concentrations of gangliosides and aggregated A β s (A β 1-40 or A β 1-42) on embryonic NSC. The number of NSC on monolayer culture in the presence of gangliosides (0.001, 0.01, 0.1, 1 or 10 μ M of GM1 or GM3) and aggregated A β s (10 μ M) was estimated by WST-8 assay. **a** A β 1-40, **b** A β 1-42. The relative absorbance of the WST-8 assay revealed the percentage of absorbance against vehicle treatment. The *bar* denotes SEM of 6 independent experiments (n = 6). **p* < 0.05: Comparison was made between vehicle versus each treatment. ***p* < 0.05: Comparison was made between A β only (0 μ M of ganglioside, *white column* versus each treatment

Fig. 5.

Apoptosis of NSCs treated with Aβs. Apoptotic cells in embryonic NSCs treated with soluble Aβs (10 μM of Aβ1–40 or Aβ1–42) for 3 days were detected with the TUNEL assay. The bar denotes mean ± SEM of 9 independent experiments (n = 9). Comparison was made between vehicle (0 μM of Aβ) versus each treatment. **p* < 0.05