β-Amyloid-Induced Synthesis of the Ganglioside Gd3 Is a Requisite for Cell Cycle Reactivation and Apoptosis in Neurons

Agata Copani,[1] Daniela Melchiorri,[3] Andrea Caricasole,[3] Francesca Martini,[4] Patrizio Sale,[4] Roberto Carnevale,[4] Roberto Gradini,[4] Maria Angela Sortino,[2] Luisa Lenti,[4] Ruggero De Maria,[5] and Ferdinando Nicoletti[3,6]

Departments of [1]Pharmaceutical Sciences and [2]Experimental and Clinical Pharmacology, University of Catania, 95125 Catania, Italy, Departments of [3]Human Physiology and Pharmacology and [4]Experimental Medicine and Pathology, University of Rome "La Sapienza," 00185 Rome, Italy, [5]Laboratory of Hematology and Oncology, Istituto Superiore di Sanità, 00185 Rome, Italy, and [6]I.N.M. Neuromed, 86077 Pozzilli, Italy

We have shown that cortical neurons challenged with toxic concentrations of β-amyloid peptide (βAP) enter the S phase of the cell cycle before apoptotic death. Searching for a signaling molecule that lies at the border between cell proliferation and apoptotic death, we focused on the disialoganglioside GD3. Exposure of rat cultured cortical neurons to 25 μM βAP(25–35) induced a substantial increase in the intracellular levels of GD3 after 4 hr, a time that precedes neuronal entry into S phase. GD3 levels decreased but still remained higher than in the control cultures after 16 hr of exposure to βAP(25–35). Confocal microscopy analysis showed that the GD3 synthesized in response to βAP colocalized with nuclear chromatin. The increase in GD3 was associated with a reduction of sphingomyelin (the main source of the ganglioside precursor ceramide) and with the induction of α-2,8-sialyltransferase (GD3 synthase), the enzyme that forms GD3 from the monosialoganglioside GM3. A causal relationship between GD3, cell-cycle activation, and apoptosis was demonstrated by treating the cultures with antisense oligonucleotides directed against GD3 synthase. This treatment, which reduced βAP(25–35)-stimulated GD3 formation by ~50%, abolished the neuronal entry into the S phase and was protective against βAP(25–35)-induced apoptosis.

Key words: Alzheimer's disease; β-amyloid; cell cycle; ganglioside GD3; apoptosis; neurodegeneration

It is currently believed that neuronal degeneration in Alzheimer's disease (AD) is caused by extracellular β-amyloid peptide (βAP) (for review, see Selkoe, 2001). Cultured neurons exposed to βAP predominantly show an apoptotic phenotype (Forloni et al., 1993; Loo et al., 1993), although neuronal apoptosis by β-amyloid is not the only factor that contributes to the pathophysiology of AD (Behl, 2000; Mattson, 2000; Joseph et al., 2001; Roth, 2001; Small et al., 2001). Molecular determinants of βAP-induced neuronal death have been investigated extensively, but they are still poorly defined. *In vivo* and *in vitro* studies have shown that an untimely activation of a cell cycle in terminally differentiated neurons may be a requisite antecedent to neuronal apoptosis in AD (Herrup and Busser, 1995; Vincent et al., 1996; Arendt et al., 1998; Busser et al., 1998; Nagy et al., 1998; Copani et al., 1999; Giovanni et al., 1999, 2000; McShea et al., 1999; Yang et al., 2001). We have shown that full-length βAP (fragment 1–42) and its active fragments βAP(1–40) and βAP(25–35) promote the activation of a cell cycle in differentiated cultured cortical neurons. In particular, βAP-treated cortical neurons express the repertoire of proteins necessary to exit quiescence and eventually enter S phase. These neurons undergo apoptosis before entering the G_2/M phase (Copani et al., 1999).

Because βAP-induced activation of this "neuronal cycle" seems to be critical for the development of apoptosis, it becomes important to disclose the signaling pathway(s) leading to reactivation of the cell cycle in neurons.

Gangliosides, sialic acid-containing glycosphingolipids, constitute a signaling system involved in the modulation of processes of neuronal proliferation and differentiation. GD3 is highly expressed in the embryonic nervous system, particularly in neuroprogenitor cells. GD3 levels are low in the adult brain (Percy et al., 1991; Svennerholm et al., 1991; Goldman and Reynolds, 1996; Kawai et al., 1998), although they increase in the brains of patients with AD (Kalanj et al., 1991) and Creutzfeldt–Jakob disease (Ando et al., 1984). Interestingly, endogenous GD3 neosynthesis is associated with the appearance of a tumor phenotype in melanocytes (Birkle et al., 2000), and overexpression of GD3 synthase (the α-2,8-sialyltransferase that generates GD3 from GM3) enhances the proliferation rate of both rat C6 glioma (Sottocornola et al., 1998) and PC12 pheochromocytoma cell lines (Fukumoto et al., 2000). Thus, it appears that GD3 is able to modify the cell proliferation status under physiological and pathological conditions.

In the present study we show that mature rat cortical neurons in culture, which respond to βAP by re-entering the cell cycle, show an early increase in the intracellular levels of GD3. GD3 synthesis in βAP-treated neurons is required for their entrance into the S phase and contributes to the development of apoptosis.

MATERIALS AND METHODS

Pure neuronal culture. Cultures of pure cortical neurons were obtained from rats at embryonic day 15 as described previously (Copani et al., 1999). Briefly, dissociated cortical cells were plated in a medium consisting of DMEM/Ham's F12 (1:1) supplemented with the following components: 10 mg/ml bovine serum albumin, 10 μg/ml insulin, 100 μg/ml transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, 2

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Correspondence should be addressed to Dr. Agata Copani, Department of Pharmaceutical Sciences, University of Catania, Viale A. Doria 6, 95125 Catania, Italy. E-mail: acopani@katamail.com.

mM glutamine, 6 mg/ml glucose, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cortical cells were plated at a density of 2×10^6 cells/dish on 35 mm Nunc (Roskilde, Denmark) dishes precoated with 0.1 mg/ml poly-D-lysine. Cytosine-β-D-arabinofuranoside (10 μM) was added to the cultures 18 hr after plating to avoid the proliferation of non-neuronal elements and was kept for 3 d before medium replacement. This method yields >99% pure neuronal cultures, as judged by immunocytochemistry for glial fibrillary acidic protein and neuron-specific microtubule-associated protein 2 (Copani et al., 1999). βAP has always been applied to mature cultures at 8 d *in vitro* (DIV).

Handling of β-amyloid peptide. βAP(25–35) and the reverse peptide βAP(35–25) were purchased from Bachem AG (Bubendorf, Switzerland). Different lots of peptides were used. βAP(25–35) and βAP(35–25) were solubilized in sterile, doubly distilled water at an initial concentration of 2.5 mM and stored frozen at −20°C. They were used at a final concentration of 25 μM in the presence of the glutamate receptor antagonists MK-801 (10 μM) and DNQX (30 μM) to prevent the excitotoxicity mediated by endogenous glutamate (Copani et al., 1999).

Addition of antisense oligonucleotides to the cultures. Cultures were also treated with the following "end-capped" phosphorothioate antisense oligonucleotides directed against the enzyme α-2,8-sialyltransferase (GD3 synthase): 5′-CAGTACAGCCATGGCCCCTCT-3′. A scrambled oligonucleotide was used as a control: 5′-CGACCTACCTATGCGCT-ACCG-3′. Oligonucleotides (3 μM) were applied to the cultures 16 hr before the addition of βAP(25–35).

Fluorescence-activated cell sorter analysis. Fluorescence-activated cell sorter analysis was performed as described previously (Copani et al., 1999). Cells were harvested by incubation with 0.25% trypsin for 3 min, and the suspension was centrifuged at low speed after addition of 50% fetal calf serum. Each pellet was washed with PBS and finally fixed in 70% ethanol. Before staining with propidium iodide (50 μg/ml in the dark for 30 min), suspended cells were treated for 1 hr at 37°C with RNase (100 μg/ml). The DNA content and ploidy were assessed using a Coulter Elite flow cytometer (Beckman, Fullerton, CA). The Multicycle AV software program (Phoenix Flow Systems, San Diego, CA) was used to analyze cell-cycle distribution profiles.

Evaluation of sphingomyelin hydrolysis. Cortical neurons were incubated in the presence of [^{3}H]serine (specific activity, 26 Ci mmol^{-1}; Amersham Biosciences, Milan, Italy) for 72 hr before exposure to βAP(25–35) for 4 hr. The reaction was stopped by adding methanol: chloroform:HCl (100:100:1, v/v/v) and a balanced salt solution containing 10 mM EDTA; the aqueous and lipid phases were separated by centrifugation. Glycerophospholipids present in the lipid phase were saponified in methanolic KOH (0.1 M for 1 hr at 37°C) before resolution of sphingomyelin by sequential one-dimensional TLC, using chloroform: benzene:ethanol (80:40:75, v/v/v) followed by chloroform:methanol:28% ammonia (65:25:5, v/v/v) as solvents. Plates were analyzed using a digital autoradiographer (Berthold, Bad Wildbad, Germany).

Assessment of intracellular GD3 levels. Cultures were washed twice with ice-cold PBS, pH 7.4, and cells were scraped from the dishes and homogenized. Gangliosides were extracted according to the method of Svennerholm and Fredman (1980) as described previously (Dotta et al., 1998) and analyzed by high-performance TLC (HPTLC) using analytical precoated Silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). All plates were first activated by heating to 100°C for 30 min. Samples were spotted onto plates with a Hamilton syringe in chloroform:methanol:0.25% KCl (5:4:1, v/v/v). Authentic GD3 (provided by Fidia S.p.A., Abano Terme, Italy) was used as standard. GD3 was immunodetected by using the R24 anti-GD3 monoclonal antibody (1:100). The plates were incubated for 1 hr at room temperature with the primary antibody, washed twice with PBS–Tween 20, and then incubated for 45 min at room temperature with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (1:200; Sigma, St. Louis, MO). Detection was performed by ECL (Amersham Biosciences). The bands were quantified by scanning densitometric analysis.

Immunofluorescence analysis of GD3. Cultures were fixed with 2% paraformaldehyde. After incubation with 3% nonimmunized mouse serum in PBS, the R24 anti-GD3 monoclonal antibody (1:100) was applied at 4°C for 72 hr. Cells were washed three times and FITC-conjugated anti-mouse Ig (1:200; Cappel, ICN Biomedicals, Milan, Italy) was applied for 1 hr at room temperature to visualize the labeled sites. Nuclei were stained with propidium iodide (50 μg/ml) in PBS. Fluorescence was detected by a Zeiss (Oberkochen, Germany) LSM510 laser scanner microscope.

Reverse transcriptase-PCR analysis of GD3 synthase. Total RNA was extracted from cultures of primary cortical neurons essentially as described previously (Auffray and Rougeon, 1980), except that cells were washed twice with ice-cold PBS and then scraped in 2 ml of cold 3 M LiCl/6 M urea and the procedure was scaled down appropriately. Finally, total RNA was subjected to DNase I treatment (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Two micrograms of total RNA were then used for cDNA synthesis, using Superscript II (Invitrogen, San Diego, CA) and an oligo(dT) primer according to the manufacturer's instructions. The reverse transcriptase (RT) product was diluted to 100 μl with sterile, distilled water, and 1 μl of cDNA was used in each subsequent PCR amplification. Amplification of GD3 synthase cDNA was performed using the following primers: forward (5′-CCAGCATAATTCGCCAGAGA-3′) and reverse (5′-TTGCATGTTCACGGAGAAGG-3′). For β-actin cDNA amplification, the primers were those described by Roelen et al. (1994), which span an intron and yield products of different sizes depending on whether cDNA or genomic DNA is used as a template (400 bp for a cDNA-derived product and 600 bp for a genomic DNA-derived amplification). Reaction conditions included an initial denaturation step (94°C for 3 min) followed by 45 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. A final extension step (72°C for 10 min) concluded the reaction. PCR products (one-third of the reaction) were analyzed electrophoretically on 2% agarose gels poured and run in 1× Tris acetate-EDTA.

RESULTS

We have shown previously that βAP(1–42) or its active fragments βAP(1–40) and βAP(25–35) reactivate the cell cycle and induce apoptotic death in pure cultures of cortical neurons (Copani et al., 1999). Because identical effects were seen with the three peptides,

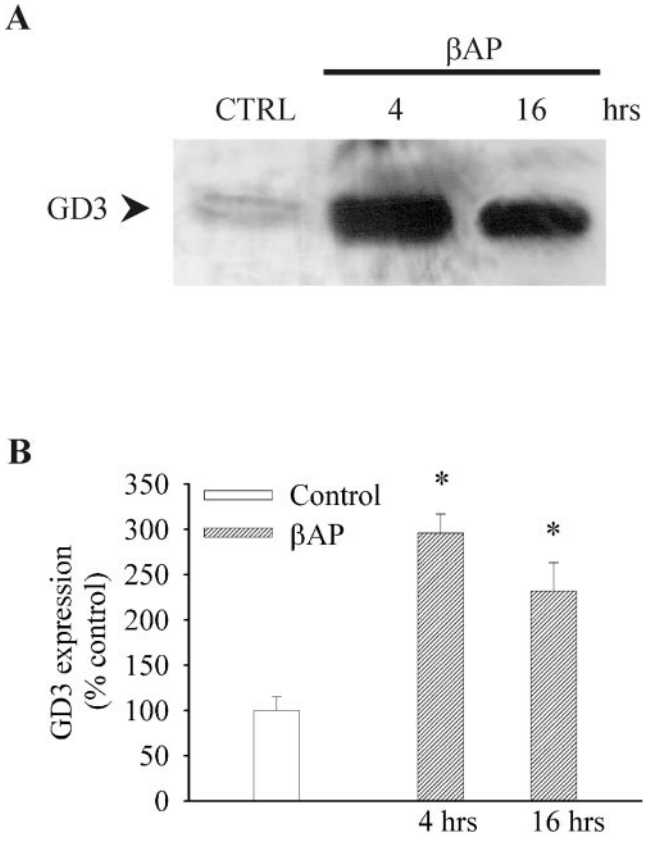


Figure 1. Intracellular GD3 levels in cultured cortical neurons exposed to βAP(25–35) for 4 or 16 hr. *A*, A representative TLC stained with anti-GD3 antibodies. *B*, A densitometric analysis of three independent experiments. Four culture dishes (2×10^6 neurons per dish) were pooled for each condition in any experiment. Values are expressed as percentages of controls and represent means ± SEM. *$p < 0.05$ (one-way ANOVA plus Fisher's least significant difference) versus controls.

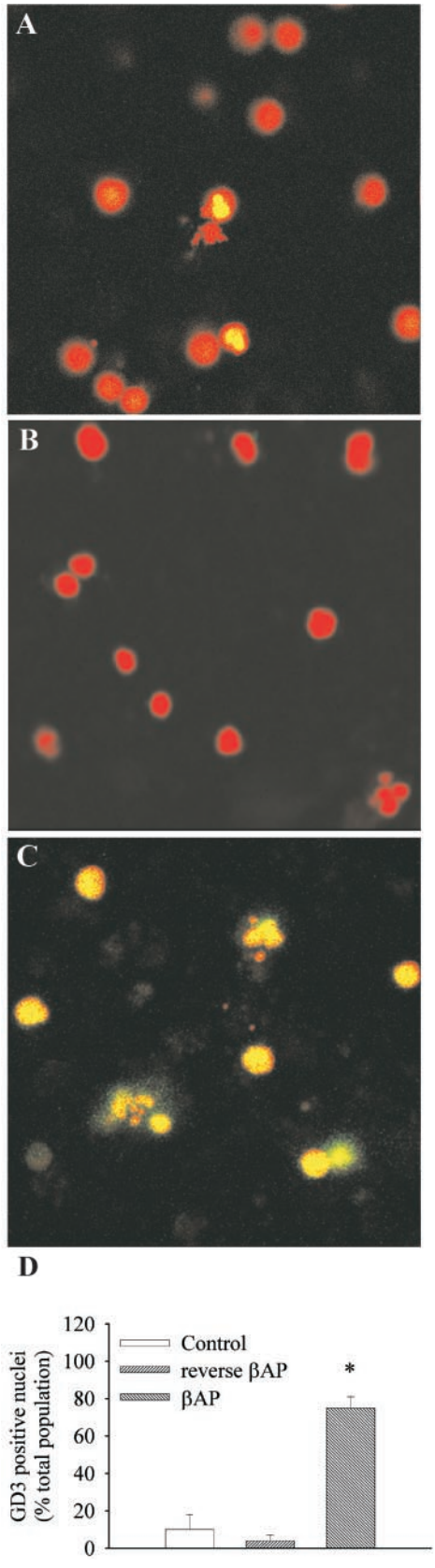


Figure 2. Immunofluorescence analysis of GD3 in control cultures (*A*) and in cultures exposed to reverse βAP(35–25) (*B*) or to βAP(25–35) (*C*) for 16 hr. GD3 immunofluorescence is in *green*. Fluorescence staining of DNA with propidium iodide is in *red*. Colocalization between GD3 and DNA is in *yellow*. The count of GD3-positive nuclei is shown in *D*. Values were calculated by a blinded observer from three random fields per culture dish for a total of four to six culture dishes per condition in two to three independent experiments. Each single dish has been considered as an individual value in the statistical analysis (i.e., $n = 4–6$). Values are means ± SEM. $^*p < 0.05$ (one-way ANOVA plus Fisher's least significant difference) versus controls or reverse βAP(35–25). Reverse βAP(35–25) was not toxic in these experiments.

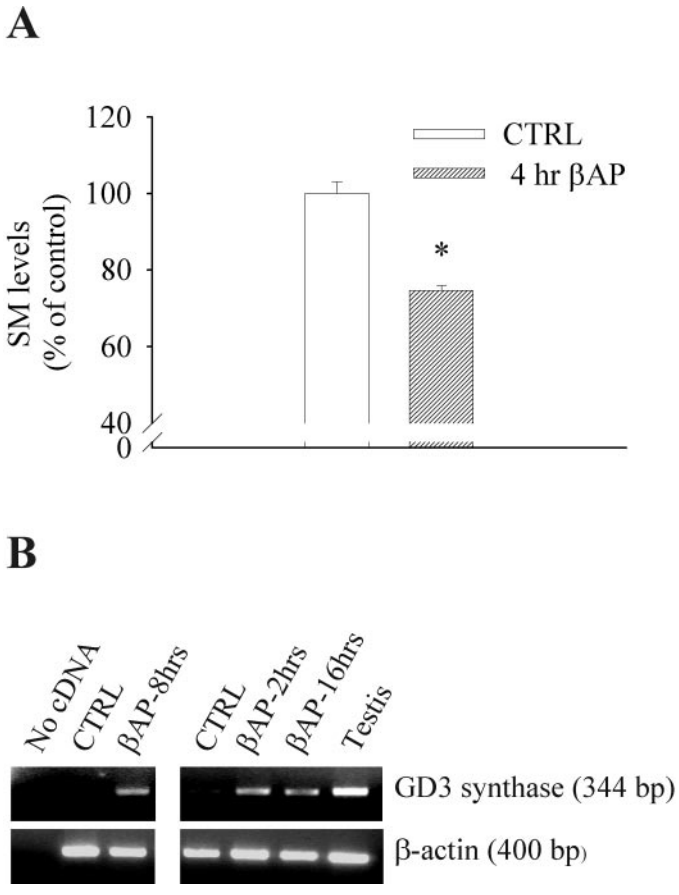
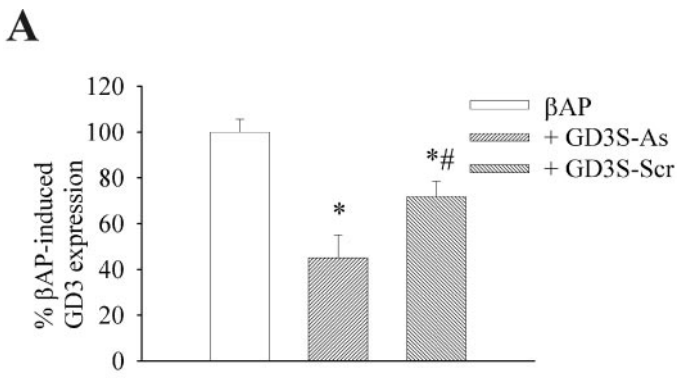
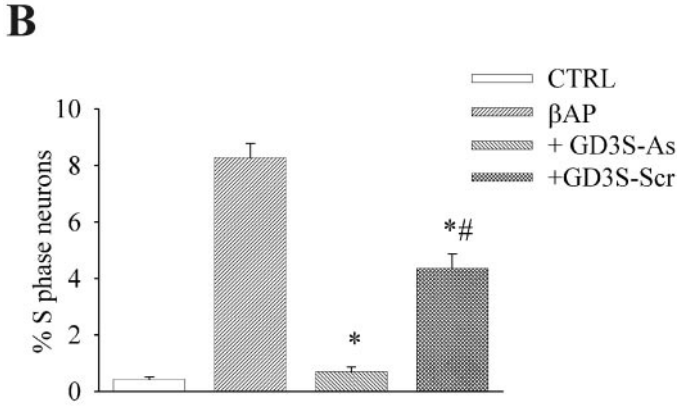


Figure 3. *A*, Exposure of cultured cortical neurons to βAP(25–35) for 4 hr reduces sphingomyelin (*SM*) levels. Values are means ± SEM of five individual determinations. $^*p < 0.01$ (Student's *t* test) compared with control cultures. *B*, Expression of GD3 synthase in primary cultures of rat cortical neurons exposed to βAP(25–35) for the indicated times. The results of two representative experiments are shown. *CTRL*, Control cultures. Amplification of β-actin cDNA was performed to confirm the integrity of the cDNA preparations and to control for genomic DNA contamination. The 600 bp of β-actin was not detected, thus excluding any genomic contamination.

we used βAP(25–35) in the present study. βAP(25–35) (25 μM) was applied to mature cultures (8–9 DIV) in the presence of a mixture of ionotropic glutamate receptor antagonists (10 μM MK-801 plus 10 μM DNQX) to avoid any endogenous excitotoxic component (Copani et al., 1999). As expected, ~8–10% of cultured neurons were found in S phase 16 hr after the addition of βAP(25–35), whereas no S phase was seen at earlier times (4 or 8 hr). The number of apoptotic neurons increased progressively after 16 hr, reaching >50% of the cell population at 20 hr (see also Copani et al., 1999).

TLC analysis combined with immunodetection showed a large increase in GD3 content 4 hr after the addition of βAP(25–35) (i.e., at a time that precedes both neuronal entry into S phase and





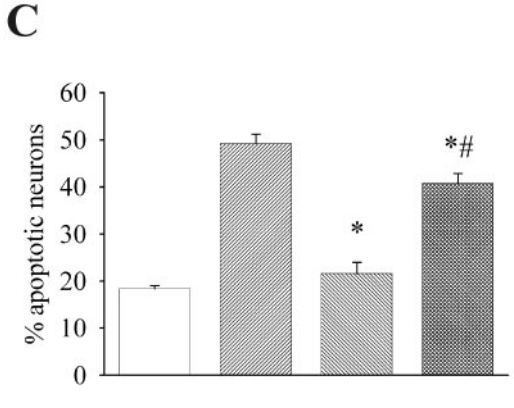


Figure 4. Intracellular GD3 levels (*A*), percentage of neurons in the S phase of the cell cycle (*B*), and percentage of apoptotic neurons (*C*) in cultured cortical neurons pretreated for 16 hr with GD3 synthase antisense oligonucleotides (*GD3S-As*, 3 μM) or a scrambled oligonucleotide (*GD3S-Scr*, 3 μM), and then exposed to βAP(25–25) for 4 hr (*A*) or 20 hr (*B, C*). Densitometric analysis of GD3 levels from three independent experiments is shown in *A*. Four culture dishes (2×10^6 neurons per dish) were pooled for each condition in any experiment. Values in *B* and *C* were calculated from eight individual culture dishes from three independent experiments. Each single dish has been considered as an individual value in the statistical analysis (i.e., $n = 8$). $p < 0.05$ (one-way ANOVA plus Fisher's least significant difference) if compared with cultures treated with βAP(25–35) alone (*) or with βAP plus GD3S-As (#).

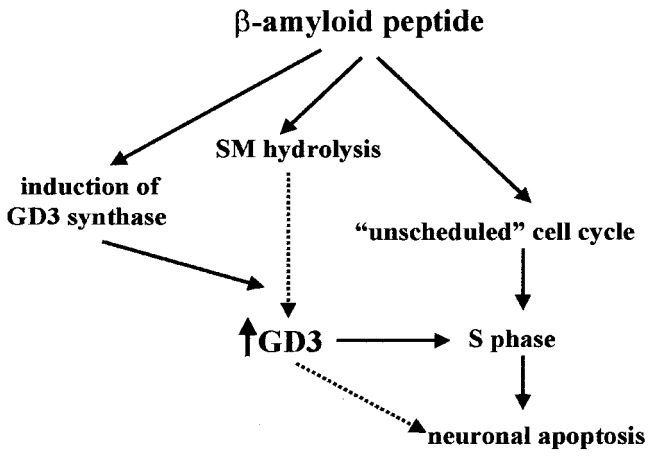


Figure 5. Hypothetical model of the role of GD3 in βAP-induced cell-cycle activation and apoptosis. *SM*, Sphingomyelin.

apoptotic death) (Fig. 1). GD3 levels decreased, but were still higher than in control cultures, 16 hr after the addition of βAP(25–35) (Fig. 1*A,B*).

In Figure 2*A,B,D*, double-fluorescence analysis of GD3 (*green*) and nuclear chromatin (*red*) showed few neurons expressing GD3 in control cultures or in cultures exposed to the reverse peptide βAP(35–25). Nearly all neurons became immunopositive for GD3 in neurons exposed to βAP(25–35). Immunoreactivity was mostly detected in cell nuclei (*yellow*) after the addition of βAP(25–35), although it was also found outside the nuclear region in late degenerating neurons (Fig. 2*C,D*).

The early increase in GD3 expression paralleled a reduction of the sphingomyelin content in neurons exposed to βAP(25–35) for 4 hr (Fig. 3*A*), suggesting that GD3 is synthesized after βAP(25–35)-induced sphingomyelin hydrolysis. RT-PCR analysis showed that βAP(25–35) induced the expression of GD3 synthase mRNA after 2, 8, and 16 hr (Fig. 3*B*).

To examine whether the increase in GD3 levels was causally related to the reactivation of the cell cycle and apoptotic death induced by βAP, we treated the cultures with a 3 μM concentration of end-capped antisense oligonucleotides directed against GD3 synthase for 16 hr before the addition of βAP(25–35). GD3 synthase antisense oligonucleotides substantially reduced the rise of neuronal GD3 content induced by βAP(25–35) at 4 hr, whereas a scrambled oligonucleotide had a smaller effect (Fig. 4*A*). Cytofluorometric analysis showed that GD3 synthase antisense oligonucleotides abolished the neuronal re-entry into the S phase of the cell cycle in response to βAP(25–35) (Fig. 4*B*) and substantially protected against βAP(25–35)-induced apoptosis (Fig. 4*C*). Scrambled oligonucleotides could also reduce βAP(25–35)-induced S phase and apoptosis, but their effect was much smaller and was significantly different from that produced by GD3 synthase antisense oligonucleotides (Fig. 4*B,C*).

Together, these results indicate that βAP(25–35)-induced GD3 synthesis is antecedent and causally related to neuronal cell-cycle reactivation and apoptosis.

DISCUSSION

After the induction of apoptosis, GD3 accumulates in non-neuronal cells, where it contributes to the death pathway by a dual mechanism that involves the opening of mitochondrial permeability transition pores and the suppression of the nuclear factor-κB-dependent survival pathway (De Maria et al., 1997; Kristal and

Brown, 1999; Malisan and Testi, 1999; Scorrano et al., 1999; Rippo et al., 2000; Colell et al., 2001). In neurons, a number of studies have been performed with exogenously added gangliosides (Favaron et al., 1988; Manev et al., 1990; Saito et al., 1998, 1999; Ryu et al., 1999), but the functional role of endogenously generated gangliosides in neurodegenerative processes has never been investigated.

In this study, we show that GD3 accumulates in rat cortical neurons that have been exposed to βAP(25–35). We have demonstrated previously that mature neurons must re-enter the cell cycle and cross the G_1/S transition before undergoing βAP-induced apoptosis (Copani et al., 1999). βAP-treated cortical neurons express a battery of proteins that are typically expressed by proliferating cells during G_1/S phases, such as cyclin D1, phosphoretinoblastoma, cyclin E, and cyclin A. Neurons eventually enter the S phase, as shown by quantitative cytofluorometric analysis, and then die by apoptosis before crossing the border between the S and G_2 phases (Copani et al., 1999). The use of a dexamethasone-inducible cyclin D1 mRNA antisense, a negative dominant mutant of cyclin-dependent kinase type 2 (CDK2) or chemical CDK inhibitors has shown that the unscheduled cell cycle is causally related to apoptotic death in neurons exposed to βAP (Copani et al., 1999, 2001). These *in vitro* studies have their *in vivo* counterpart in the AD brain, in which Yang et al. (2001) provided evidence for chromosomal replication in "at-risk" neurons. The present data indicate that GD3 is an essential component of the signaling pathway(s) leading to the reactivation of the cell cycle in βAP-treated neurons (Fig. 5). This evidence is consistent with the regulatory functions of GD3 in cellular proliferation and differentiation processes. PC12 cells overexpressing the GD3 synthase gene showed an enhanced rate of proliferation attributable to a sustained activation of the Ras/mitogen-activated protein–extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway and failed to differentiate in response to NGF (Fukumoto et al., 2000). In malignant cells, GD3 induces the suppression of differentiation phenotypes and promotes proliferation (Sottocornola et al., 1998; Birkle et al., 2000).

In neurons that degenerate in response to βAP, neosynthesized GD3 accumulated inside the nuclear region and colocalized with nuclear chromatin. In other cell types, in which apoptosis is not associated with a reactivation of the cell cycle, GD3 is instead consistently found outside the nucleus (Malisan and Testi, 1999). To date, only gangliosides of the "a" series have been described in the cell nucleus. GM1 is found in the nuclear membrane, where its expression is upregulated during axonogenesis (Wu et al., 1995, 2001; Kozireski-Chuback et al., 1999). Interestingly, GM1 inhibits DNA synthesis and the activity of DNA polymerase α in isolated nuclei of HeLa cells (Ohsawa et al., 1988). An attractive hypothesis is that GD3 acts as a functional counterpart of GM1 by increasing cell proliferation via a nuclear mechanism. Accordingly, in neurons exposed to βAP, GD3 might provide a nuclear signal for the aberrant DNA replication. The evidence that the antisense-induced decrease in GD3 levels was highly effective in preventing the unscheduled S phase, and the ensuing apoptotic phenotype strengthens the hypothesis of a causal relationship among GD3 formation, cell-cycle activation, and neuronal death. However, we cannot exclude the possibility that GD3 contributes to βAP-induced apoptosis through additional mechanisms (Fig. 5), for example by targeting the mitochondrial pathway of cell death.

Ceramide released from sphingomyelin hydrolysis is a likely source for GD3 synthesis (De Maria et al., 1998). Sphingomyelin hydrolysis might follow the interaction of βAP aggregates with a membrane receptor, the identity of which is unknown. The p75 low-affinity neurotrophin receptor is a possible candidate (Yaar et al., 1997), because this receptor has been shown to transduce the extracellular signal via the activation of acidic sphingomyelinase (Brann et al., 1999; Bilderback et al., 2001). The finding of an early induction of GD3 synthase in response to βAP is particularly interesting because it provides the first evidence that this enzyme is upregulated in response to a death signal. This suggests a novel strategy against βAP-induced neurotoxicity based on the pharmacological regulation of GD3 synthase expression.

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