

An early increase in the disialoganglioside GD3 contributes to the development of neuronal apoptosis in culture

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Abstract

We induced apoptosis in primary cultures of cerebellar granule neurons by switching the growing medium into a medium containing lower concentrations of K⁺ (5 or 10 mM instead of 25 mM) or, alternatively, by addition of staurosporine. The apoptotic phenotype was always preceded by an early increase in the intracellular levels of the disialoganglioside GD3, which peaked at 2–6 h and returned back to normal at 12 h. GD3 synthase, the enzyme that forms GD3 from the monosialoganglioside GM3, was also induced at early times after the induction of apoptosis in granule cells. Immunofluorescent staining showed that GD3 increased in neuronal cell bodies and neurites, but was never localized in cell nuclei. In cultures switched into a low K⁺-containing medium, exogenously applied GD3, but not the disialoganglioside GD1a, accelerated the development of neuronal apoptosis. In contrast, the antisense-induced knock-down of GD3 synthase was protective against granule cell death induced by lowering extracellular K⁺ from 25 to 10 – but not 5 – mM. These results demonstrate that an early and transient increase in GD3 synthesis is one of the factors that contribute to the induction of neuronal apoptosis in culture.

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Keywords: GD3; cerebellar granule neurons; apoptosis; neuronal cultures

Abbreviations: DIV, days *in vitro*; NF- κ B, nuclear factor- κ B; s.c., serum containing; s.f., serum-free; HPTLC, high performance thin layer chromatography; PBS, phosphate buffered saline

Introduction

Neuronal apoptosis is widely implicated in a variety of physiological and pathological processes, such as the elimination of supranumerary neurons during CNS development or the progressive loss or vulnerable neurons in acute or chronic neurodegenerative disorders.^{1–3} In spite of an extensive research, the molecular events that mediate the early steps of the apoptotic signal in neurons are largely unknown. In myeloid and lymphoid tumour cells, an early and transient increase in the formation of the disialoganglioside GD3 is required for the development of apoptosis in response to Fas/CD95 receptor activation.⁴ A role for GD3 in apoptosis is strengthened by the evidence that GD3 induces mitochondrial permeability transition with ensuing release of Cytochrome *c* and caspase activation.^{4–9} Alternatively, GD3 has been shown to enhance apoptosis by preventing the nuclear translocation of nuclear factor- κ B (NF- κ B), thus suppressing the NF- κ B-dependent survival pathway.¹⁰ Although gangliosides are major constituents of the vertebrate brain, the role of GD3 in neuronal apoptosis is still unexplored. We have addressed this issue in cultured cerebellar granule cells, which offer the advantage of a high homogeneity of the neuronal population. Cultured granule cells are usually grown under chronic depolarizing conditions (i.e. in medium containing 25 mM K⁺), which are required for cell maturation and survival. In these cultures, apoptosis may be induced by switching the growing medium into a medium containing lower concentrations of K⁺, independently of the presence of serum. This particular form of 'apoptosis by trophic deprivation' incorporates features of the developmental apoptosis of granule cells that physiologically occurs during early postnatal life, and has largely been used for the study of the molecular determinants of neuronal death.^{11–16} We report that the induction of apoptosis in cultured granule cells is associated with an early and transient increase in the intracellular levels of GD3, and that GD3 depletion is protective against neuronal death.

Results

Cultured cerebellar granule cells grown in medium containing 10% FCS and 25 mM K⁺ (K25) were viable and fully differentiated after 6 days *in vitro* (DIV). For the induction of apoptosis, we have switched the growing medium either into a fresh serum-free (s.f.) medium containing 5 mM K⁺ (K5) or into a conditioned medium collected from sister cultures grown in the presence of serum (serum containing or s.c.) but with 10 mM K⁺ (K10). We did not switch K25 cultures into fresh scK10 medium to avoid the induction of excitotoxic neuronal death by the micromolar amounts of glutamate present in fresh serum.¹⁷ Cultured granule cells switched from K25 into sfK5 or scK10 developed substantial apoptotic death within 48–72 h (Figure 1). No phenotypic hallmarks of

apoptosis (chromatin fragmentation or nuclear picnosis) were visible by Hoechst staining during the first 12 h following the medium switch (Figure 1). 'Low-K⁺-induced apoptosis was associated with mitochondrial membrane depolarization, as indicated by the increase in the green fluorescence of the dye JC-1 (Figure 2). Apoptosis was also induced by applying the protein kinase inhibitor, staurosporine (50 nM), to K25

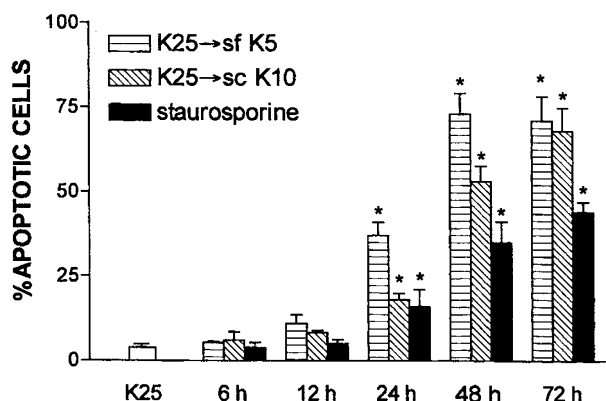


Figure 1 Development of apoptosis in cultured cerebellar granule cells treated as follows: (i) switch of the growing medium (K25) into a fresh K5 medium lacking foetal calf serum (sfK5); (ii) switch of the growing medium into a medium collected from sister cultures grown in K10 in the presence of serum (scK10); (iii) addition of 50 nM staurosporine to the growing medium. No increase in the percentage of apoptotic neurones was seen in cultures switched from K25 into K25 at any time (not shown). Apoptotic death was assessed by Hoechst staining. Values are means \pm S.E.M. of 5–6 determinations. * $P < 0.05$ (One-way ANOVA *Fisher's PLSD) as compared to the respective values at 6 and 12 h

cultures. At these concentrations, staurosporine produced apoptotic death in about 35% of neurons after 72 h (Figure 1). In all these models of trophic deprivation, the phenotypic hallmarks of apoptosis were preceded by a transient increase in the intracellular levels of GD3, as detected by HPTLC combined with immunodetection. The increase in GD3 peaked between 4 and 6 h after switching the culture medium from K25 into sfK5 or scK10, whereas it was maximal 2 h after the addition of staurosporine. GD3 levels returned back to normal after 12 h (Figure 3). HPTLC analysis performed in cultures switched from K25 into scK10 also showed an early increase in the levels of GD1b and GT1b, which are generated from GD3 metabolism (not shown). Immunofluorescence analysis carried out at different times after switching the medium from K25 into sfK5 (Figure 4) or scK10 (not shown) showed that the increase in GD3 (green in Figure 4) was localized both in the cell bodies and neurites of granule cells. The absence of co-localization between GD3 immunofluorescence and propidium iodide (red in Figure 4) indicated that GD3 accumulated outside the cell nucleus. GD3 was nearly undetectable in K25 control cultures (Figure 4). Apoptosis was also associated with an early induction of the enzyme GD3 synthase, which was detected at 2 and 4 h after switching the medium (Figure 5).

To examine whether GD3 had any role in the induction of apoptosis, we firstly applied exogenous GD3 to the cultures. Addition of GD3 (up to 100 μ M) to K25 cultures had no effect on granule cells viability. However, when applied to the cultures immediately after switching the medium from K25 into scK10, GD3 (10 μ M) accelerated the development of apoptosis in granule cells. In the presence of GD3, an increase in the percentage of

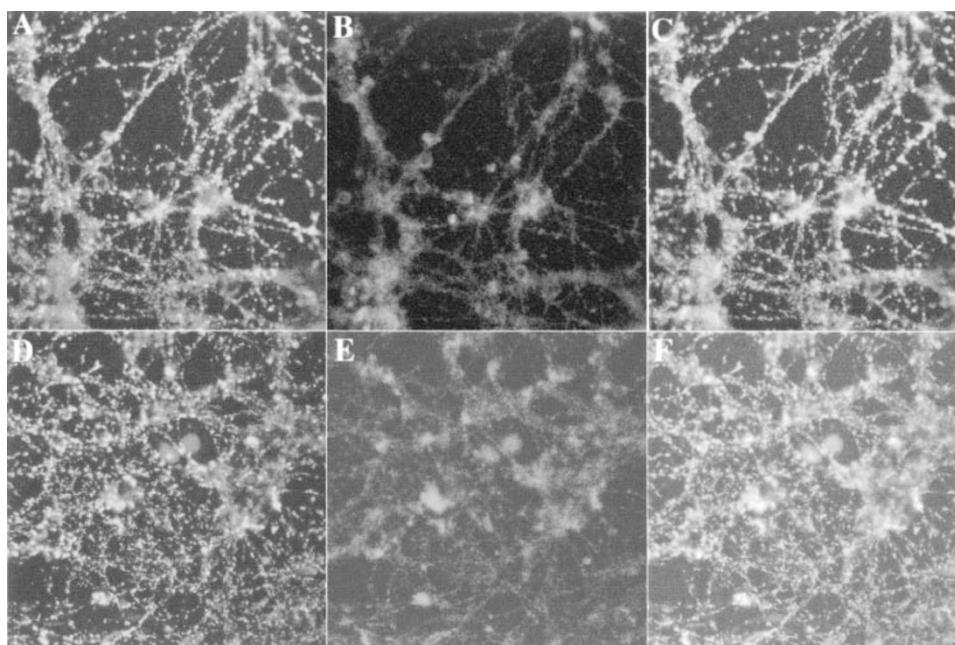


Figure 2 Changes in mitochondrial membrane potential 12 h after switching the medium of cultured granule cells from K25 into scK10. Cultures were stained with the mitochondrial dye, JC-1, which emits red fluorescence in its aggregate form with high mitochondrial membrane potentials and green fluorescence in its monomeric form with low mitochondrial membrane potentials. Fluorescence in control cultures (switched from K25 into K25) and in cultures switched from K25 into scK10 are shown in (A) and (B), and (D) and (E), respectively. Co-localization is shown in (C) and (F), respectively

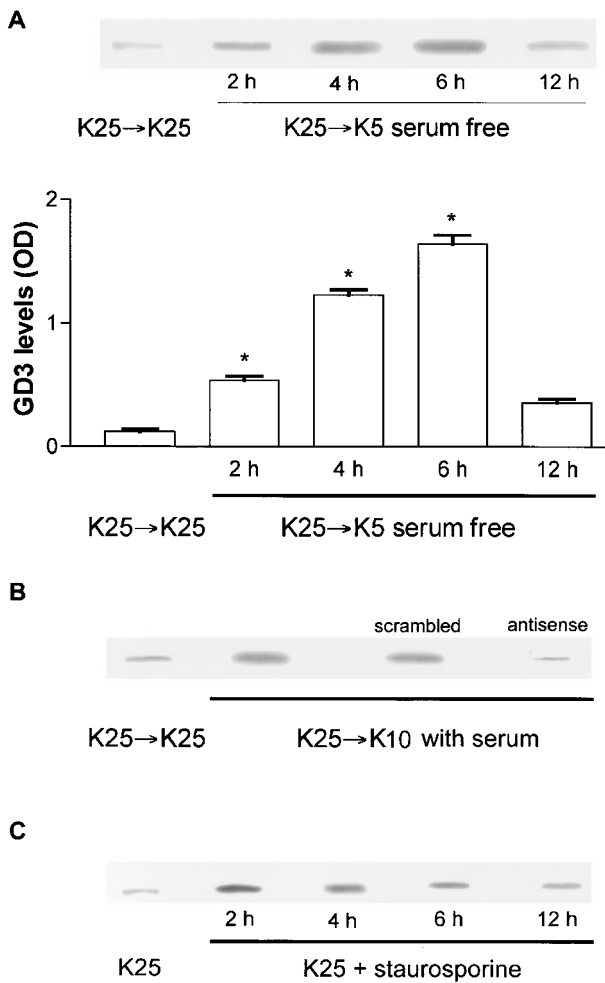


Figure 3 (A) Early increase in GD3 levels in cultured granule cells after switching the K25 medium into a K5 medium lacking foetal calf serum. Densitometric values are also shown ($n=3-4$; $*P < 0.05$ vs control values; One-way ANOVA+Fisher's PLSD). Cell number was approximately the same (2×10^6 /dish) in all dishes used for HPTLC. Values obtained 4 h after switching the K25 medium in K25 medium collected from sister cultures are shown in the first lane and considered as controls. These 'control' values did not differ from those obtained from K25 cultures that did not undergo the medium switch (compare the first lane of (A) and (C)). There was no change in cell viability and cell number up to 12 h after switching the medium from K25 into sfK5. (B) Representative HPTLC of GD3 in cultured cerebellar granule cells, 4 h after switching the K25 medium into a scK10 medium collected from sister cultures. The effect of a 2-day treatment with a GD3 antisense or a scrambled oligonucleotides (both at $2 \mu\text{M}$) is also shown. Cultures switched from K25 into K25 (4 h) are shown as controls. (C) Time-dependent increase in GD3 levels in K25 cultures treated with 50 nM staurosporine. Experiments in (B) and (C) were repeated two times with similar results

apoptotic neurons was detected after 4, 24 and 48 h after the medium switch. No increase in granule cell apoptosis was induced by GD1a, a disialoganglioside that belongs to the 'a' series (Figure 6). We also examined the role of endogenous GD3 by treating the cultures with end-capped antisense oligonucleotides directed against GD3 synthase, the enzyme that converts GM3 into GD3. K25 Cultures were treated once daily for 2 days with antisenses ($2 \mu\text{M}$) or with a scrambled oligonucleotide

($2 \mu\text{M}$), and then were switched into scK10 for the assessment of GD3 levels (after 4 h) or apoptotic death (after 48 h). In cultures tested for apoptotic death, antisense or scrambled oligonucleotides were applied once more immediately after the medium switch. Antisense treatment completely abolished the early increase in GD3 levels (Figure 3B), and significantly reduced the extent of apoptotic neuronal death in cultures switched from K25 into scK10 (Figure 7). GD3 antisenses were not protective when a more substantial apoptosis was induced by switching the medium from K25 into sfK5 (not shown).

Discussion

GD3, which is synthesized from GM3 by the enzyme α -2,8-sialyltransferase (GD3 synthase), is the metabolic precursor of gangliosides of the 'b' series (such as GD1b, GT1b and GQ1b). GD3 is heavily expressed in the embryonic nervous system and, particularly, in rapidly proliferating neuroprogenitor cells. GD3 levels decline substantially at late stages of CNS development and are low in post mitotic-neurones.¹⁸⁻²¹ Interestingly, GD3 increases in the brain of patients with Alzheimer's disease,²² Creutzfeldt-Jakob disease, and subacute sclerosis panencephalitis,²³ although the significance of this increase is obscure. GD3 levels seem to correlate with cell proliferation, as suggested by the evidence that over-expression of GD3 synthase in PC12 cell enhances the proliferation rate via a sustained activation of TrkA and ERK1/2,²⁴ and that a decreased proliferation is observed in melanoma AbC1 cells depleted of GD3.²⁵ However, an enhanced GD3 formation can also induce neuritic growth and cholinergic differentiation in Neuro2a cells.²⁶ In our experiments, cultured cerebellar granule cells grown in K25 at 6 DIV, which were viable and fully differentiated, had low levels of both GD3 and GD3 synthase. In these cultures, GD3 was mainly localized in the few astrocytes present as contaminants (not shown; see refs.^{27,28}). Interestingly, GD3 expression in granule cells was markedly increased a few hours after the induction of apoptosis. Apoptosis was induced by switching the medium from K25 into sfK5 or scK10, or, alternatively, by applying the broad protein kinase inhibitor, staurosporine. In all models, the increase in GD3 peaked 2-4 h after the induction of apoptosis, i.e. many hours before the appearance of the apoptotic phenotype. Therefore, an increase in GD3 formation represents an early event in the development of neuronal apoptosis. GD3 appeared to be localized in the cell body and neurites and was never detected in the nuclei of granule cells committed to death. GD3 may contribute to the apoptotic program by disrupting the mitochondrial transmembrane potential leading to the release of Cytochrome c and activation of caspase 9, as shown in other cellular models.⁴⁻⁶ The change in mitochondrial membrane potential observed in granule cells undergoing apoptosis is consistent with this hypothesis. The levels of GD3 synthase, the enzyme that forms GD3 from GM3, increased 2 and 4 h after switching the cultures from the K25 medium into sfK5, suggesting a *de novo* synthesis of GD3. This provide one of the few demonstrations of an induction of GD3 synthase in cells committed to death. To demonstrate

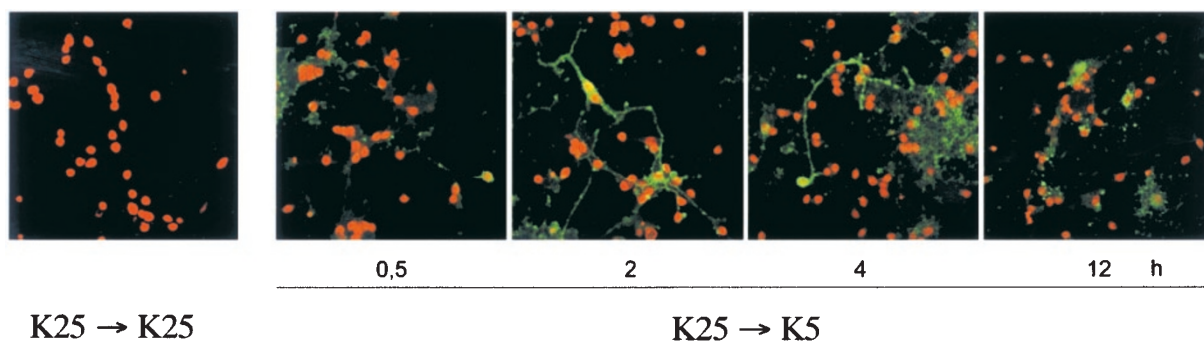


Figure 4 Immunofluorescent analysis of GD3 (green) 0.5–12 h after switching the K25 medium into a sfK5 medium. Note the lack of co-localization between GD3 and propidium iodide (red). Control cultures (12 h after switching the medium from K25 into sfK25) are also shown. A similar pattern of GD3 expression was observed in cultures switched from K25 into scK10

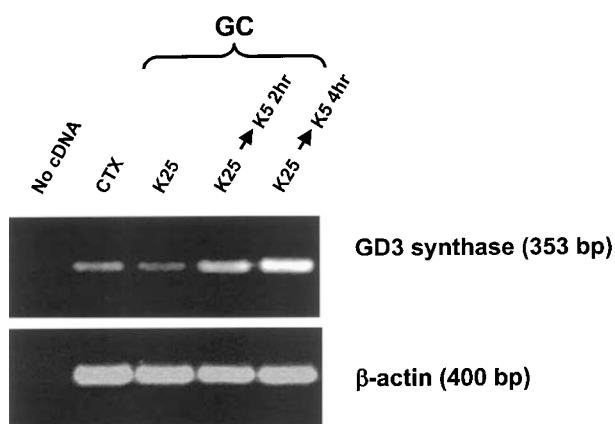


Figure 5 RT-PCR analysis of GD3 synthase mRNA in cultured granule cells (GC) switched from K25 into sfK5 (2 and 4 h). GD3 synthase in adult rat cerebral cortex (CTX) is also shown. β -Actin mRNA is shown as a control and to exclude contamination by genomic DNA (see Materials and Methods)

that the increase in GD3 formation was causally related to apoptosis we used two strategies: (i) the application of exogenous GD3 to cultures; and (ii) the depletion of endogenous GD3 by using GD3 synthase antisenses. Exogenous GD3 had no effect on the viability of K25 cultures, perhaps because the trophic input provided by chronic depolarization is strong enough to overcome an apoptotic signal. However, GD3 accelerated the development of 'low- K^{+} '-induced apoptosis and, remarkably, almost 20% of granule cells treated with GD3 acquired the apoptotic phenotype as early as 4 h after the medium switch. Such a rapid development of apoptosis strongly supports a role for GD3 in neuronal death. GD3 synthase antisenses applied to cultures switched from K25 into scK10 reduced the extent of apoptosis by about 40–50%, although it abolished early increase in GD3 levels. This suggests that an enhanced formation of GD3 contributes to, but is not the only factor involved in the induction of neuronal apoptosis. Other factors might become predominant under more severe death-inducing conditions, as observed in cultures switched from K25 into sfK5. The lack of protection by GD3 synthase antisenses in the latter condition is consistent with this view.

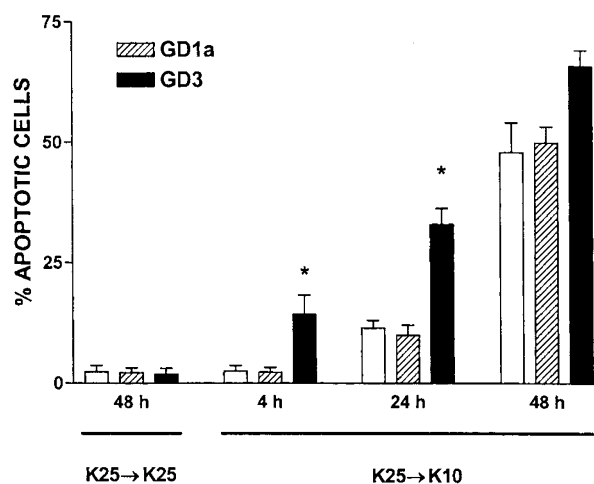


Figure 6 Effect of exogenous application of GD3 or GD1a (both at 10 μ M) on the percentage of apoptotic neurons in cultures switched from a K25 medium into a scK10 medium collected from sister cultures. In this particular experiment, the percentage of apoptotic neurons was determined by FACS analysis after DNA staining with propidium iodide. Values are means \pm S.E.M. of 3–9 determinations. * $P < 0.05$ (One-way ANOVA+Fisher's PLSD) vs values obtained from cultures switched from K25 into K10 and treated with buffer or GD1a

Perhaps GD3 is mandatory or dispensable depending on how much additional factors participate to the activation of the mitochondrial pathway of cell death. Thus, only if the intensity of the apoptotic stimulus is mild, GD3 can contribute to reach the threshold for the activation of the mitochondrial cascade leading to Cytochrome *c* release and caspase activation. However, we cannot exclude that neuroprotection deriving from the knock-down of GD3 synthase in cultures switched from K25 into scK10 depends on an increased formation of gangliosides of the 'a' series from the common precursor, GM3. Accordingly, the gangliosides GM1 and GD1a or their semisynthetic derivatives are protective against apoptosis induced by ethanol- or 'low- K^{+} ' in cultured cerebellar granule cells,^{29,30} or by serum deprivation, ionomycin or cyclosporin A in cultured cortical neurons.³¹

In conclusion, present results provide the first evidence that an early and transient increase in the GD3 ganglioside

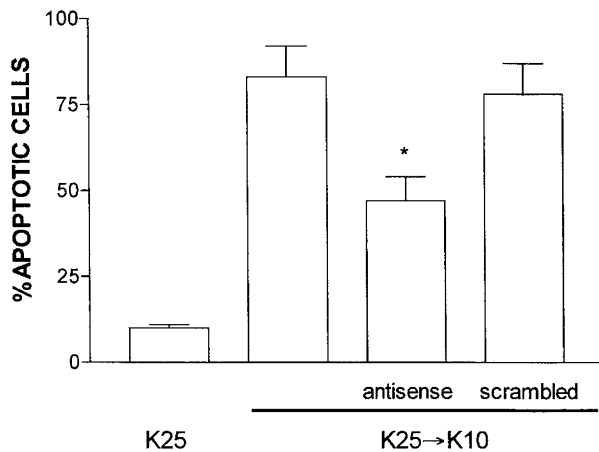


Figure 7 Antisense oligonucleotides directed against GD3 synthase reduced the extent of apoptotic death in cultured granule cells. Cultures were switched from a K25 medium into a scK10 medium collected from sister cultures. Antisenses or scrambled oligonucleotides (both at 2 μ M) were applied once a day for the 2 days preceding the medium switch, and then re-applied immediately after the switch. Apoptotic death was assessed by Hoechst staining. Values are means \pm S.E.M. of determinations from six culture dishes. * $P < 0.05$ (One-way ANOVA+Fisher's PLSD) vs values obtained from cultures switched from K25 into scK10 that did not receive the antisense

is associated with, and causally related to, the development of apoptosis in cultured neurons. The mechanism(s) underlying the increased formation of GD3 and the metabolic source for GD3 synthesis are still obscure. An increased formation of ceramide through the acidic sphingomyelinase pathway is considered as a major source for cytotoxic GD3 in blood cells following Fas/CD95 receptor activation.^{4–7} However, there is no evidence that ceramide is produced from sphingomyelin in granule cells committed to death, although granule cells develop apoptosis in response to the cell permeable ceramide analogue, C2-ceramide.^{32–34} An induction of the Fas/CD95 ligand is consistently observed in granule cells switched into a low-K⁺-containing medium,^{35–37} but whether or not Fas/CD95 ligand contributes to the development of granule cell apoptosis is matter of controversy.^{36,37} Clearly, more studies are needed for the identification of the pathway leading to GD3 synthesis in neuronal apoptosis.

Materials and Methods

Preparation of cultured granule cells

Primary cultures of cerebellar granule cells were prepared from 8-day old Sprague-Dawley rats (Charles River, Calco, Italy) rats, as described previously.³⁸ Cells were plated on 35-mm dishes (2×10^6 /dish) in 2 ml of basal Eagle's medium (Gibco) containing 10% fetal calf serum and 25 or 10 mM K⁺ as KCl (K25 and K10). Cytosine-D-arabino-furanoside (10 μ M) was added 16–18 h after plating to avoid the proliferation of non-neuronal cells. Mature cultures contained >90% of granule cells, 3–5% of GABAergic neurons and a small percentage of non-neuronal cells (mostly glial cells) as contami-

nants.³⁸ Cultures at 6–7 days *in vitro* (DIV) were used for all experiments. The culture medium was replaced with: (a) a fresh medium containing 5 mM K⁺ (K5) and lacking fetal calf serum; or (b) the medium collected from sister cultures grown in K10 containing serum since the time of plating. In control cultures, the medium was replaced with an identical medium from sister cultures grown in K25.

Assessment of apoptotic death and of changes in mitochondrial membrane potential

Apoptotic death was assessed by fluorescent chromatin staining with Hoechst 33258, as described previously.¹⁶ Apoptotic cells were considered as those showing chromatin fragmentation or nuclear picnosis. The percentage of apoptotic neurones was also determined by FACS analysis after DNA staining with propidium iodide. Cultures were washed in PBS (pH 7.4), harvested with a cell scraper, and washed twice in PBS by low-speed centrifugation. The cell pellet was incubated for 1 h at 4°C in 70% ethanol, washed again in PBS, and finally stained in the dark for 1 h with 100 μ g/ml of propidium iodide in PBS. DNA content and ploidy was assessed by using an Epics Coulter XL flow cytometer. To assess whether granule cell apoptosis was associated with changes in mitochondrial membrane potentials, we have incubated the cultures with the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide (JC-1), which is sensitive to changes in mitochondrial membrane potential.³⁹ Cultures were incubated with 3 μ M JC-1 for 20 min at 37°C and then washed in PBS. Green and red fluorescence was detected by a Zeiss laser scanner microscope (LSM510, Oberkochen, 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 Fredman⁴⁰ as described previously⁴¹ and analysed by high performance thin layer chromatography (HPTLC) using analytical pre-coated 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, vol/vol/vol). When detection of all gangliosides was required, plates were air-dried and stained with resorcinol spray reagent, which stains sialic acid-containing glycolipids. Gangliosides GM3, GM1, GD1a, GD1b and GT1b (Sigma, St. Louis, MO, USA), GM2 and GD3 (kindly provided from Fidia Research Laboratories, Abano Terme, Italy) were used as standards. In most of the experiments, we specifically detected the GD3 ganglioside using the R24 anti-GD3 monoclonal antibody (1:100). The plates were incubated for 1 h at room temperature with the primary antibody, washed twice with PBS-Tween-20, and then incubated for 45 min at room temperature with an horseradish peroxidase-conjugated rabbit anti-mouse antibody (1:200; Sigma). Detection was carried out by ECL (Amersham, Milan, Italy). The bands were quantified by scanning densitometric analysis (GS300, Hoefer Scientific Instruments, San Francisco, CA, USA).

Immunofluorescence analysis of GD3

Cultured granule cells were fixed with 2% paraformaldehyde. Following incubation with 3% non immunized mouse serum in PBS, the R24 anti-GD3 monoclonal antibody (1:100) was applied at 4°C for 72 h. Cells were washed three times and FITC-conjugated anti-mouse immunoglobulin (Cappel, 1:200) was applied for 1 h at room

temperature to visualize the labelled sites. Nuclei were stained with propidium iodide (50 $\mu\text{g/ml}$) in PBS. Fluorescence was detected by a Zeiss laser scanner microscope (LSM510, Oberkochen, Germany).

RT-PCR analysis of GD3 synthase

Total RNA was extracted from the cultures as described by Auffray and Rougenon,⁴² except that cells were washed 2 \times with ice cold PBS and then scraped in 2 ml of cold 3 M LiCl/6M Urea and the procedure was scaled down appropriately. Total RNA was finally subjected to Dnasel treatment (Boehringer Mannheim) according to manufacturer's instructions. Two μg of total RNA were then employed for cDNA synthesis, using Superscript II (BRL Life Tech.) and an oligodT primer according to manufacturer's instructions. The RT product was diluted to 100 μl with sterile, distilled water and 1 μl of cDNA was employed in each subsequent PCR amplification. Amplification of GD3 synthase cDNA was carried out employing the following primers: forward (5'-CCAGCATAATTCGCCAGAGA-3') and reverse (5'-TTGCATGTT-CACGGAGAAGG-3'). For β -actin cDNA amplification, the primers were those described by Roelen *et al.*,⁴³ which span an intron and yield products of different sizes depending on whether cDNA or genomic DNA is employed 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/3 min) followed by 45 cycles of (94°C/30 s; 55°C/30 s; 72°C/30 s). A final extension step (72°C/10 min) concluded the reaction. PCR products (1/3 of the reaction) were analyzed electrophoretically on 2% agarose gels poured and run in 1 \times TAE.

Addition of gangliosides or antisense oligonucleotides to the cultures

GD3 and GD1a were dissolved in methanol and then dried under nitrogen. The two gangliosides were then suspended in water and applied to the cultures. Cultures were also treated with the following 'end-capped' phosphorothioate antisense oligonucleotide directed against the enzyme α -2,8-sialyltransferase (GD3 synthase): 5'-CAG TAC AGC CAT GGC CCC TCT-3'. A scrambled oligonucleotide was used as a control: 5'-CGA CCT ACC TAT GCG CTA CCG-3'. Oligonucleotides were applied to cultures at 4–5 DIV once per day for 2 days. Afterwards, the culture medium was replaced with a medium collected from sister cultures grown in K25 or K10. For the detection of GD3, cultures were stopped 4 h later without any further addition of oligonucleotides. For the assessment of apoptotic death, oligonucleotides were applied once more immediately after the medium switch, and cultures were examined 48 h later.

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