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GD3–7-aldehyde is an apoptosis inducer and interacts with adenine nucleotide translocase

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ABSTRACT

We prepared GD3–7-aldehyde (GD3–7) and determined its apoptotic potential. GD3–7 proved to be more efficient to induce pro-apoptotic mitochondrial alterations than GD3 when tested on mouse liver mitochondria. GD3–7-induced mitochondrial swelling and depolarization was blocked by cyclosporin A (CsA) supporting a critical role of the permeability transition pore complex (PTPC) during GD3–7-mediated apoptosis. In contrast to GD3, GD3–7 was able to induce channel formation in proteoliposomes containing adenine nucleotide translocase (ANT). This suggests that ANT is the molecular target of GD3–7. Using a specific antiserum, GD3–7 was detected in the lipid extract of the myeloid tumor cell line HL-60 after apoptosis induction, but not in living cells. Therefore, GD3–7 might be a novel mediator of PTPC-dependent apoptosis in cancer cells.

Introduction

Intracellular GD3 formation is a consequence of pro-apoptotic signals induced by FAS/FASL [1], TNF-α [2] and β-amyloid [3]. It has various effects such as production of reactive oxygen species (ROS) [4], opening of a mitochondrial multiprotein complex, the permeability transition pore complex (PTPC) [4], release of cytochrome *C* from mitochondria [1], activation of caspases [5], and inhibition of the translocation of NFκB into the nucleus [6]. Apoptosis mediated by GD3 can be blocked by CsA and by the oncoprotein Bcl-2, two inhibitors of the PTPC-dependent permeability transition (PT) [7,8], which underlines the important role of mitochondria in the signalling of GD3-induced apoptosis. The GD3 effect is suppressed by antioxidants [9]. This suggests that GD3 oxidation may be critical for apoptosis induction. In cells, especially in certain types of tumors GD3 is frequently found along with a derivative that is 9-*O*-acetylated at the terminal sialic acid yielding 9-*O*-acetyl GD3 (acGD3) [10–12]. AcGD3 is able to suppress the pro-apoptotic effect of GD3 [13]. While 9-*O*-acetylation probably has only a limited effect on the hydrophobicity or steric conformation of GD3, it was found to block the oxidative modification of GD3 thus preventing the formation of oxidized GD3 variants [14]. The aims of this study were to compare the impact of GD3 and its derivatives for the regulation of apoptosis. We prepared GD3–7-aldehyde (GD3–7) as a highly effective pro-apoptotic variant of GD3 and addressed the question of ANT as its mitochondrial target. In addition we demonstrated the formation of GD3–7 in the human tumoral cell line HL-60 after apoptosis induction.

Materials and methods

Cell culture and induction of apoptosis. When not indicated otherwise, chemicals were from Sigma (St. Louis, MO). HL-60 cells were obtained from the Department of Immunology, University of Munich, Germany. They were maintained at a concentration of <1 × 10^6 cells/ml in RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine. To induce apoptosis a 10 mM solution of C2-ceramide in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) was added directly to cell suspensions to obtain the indicated concentrations for a 24 h culture.

Abbreviations: amu, atomic mass units; ANT, adenine nucleotide translocase; CAT, carboxyatractyloside; CsA, cyclosporin A; CypD, cyclophilin D; $\Delta\Psi_m$, mitochondrial transmembrane potential; MMP, mitochondrial membrane permeabilization; PT, permeabilty transition; PTPC, permeability transition pore complex; VDAC, voltage-dependent anion channel; 4-MUP, 4-methyl umbelliferyl phosphate; GM3, II^3NeuAc-LacCer; GD3, $II^3(NeuAc)_2$-LacCer; GD3–7, GD3–7-aldehyde; AcGD3, 9-*O*-acetyl GD3.

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The extent of apoptosis was measured using the method of Nicoletti et al. [15].

Gangliosides. GD3 and acGD3 were purified from bovine buttermilk and characterized as described [16]. Oxidized GD3 was prepared as follows: 2 mg GD3 was sonicated in 2 ml of 100 mM sodium acetate buffer pH 5.5 in an ultrasonic bath for 15 min at 0 °C. The solution was then mixed with 2 ml of 10 mM sodium periodate in the same buffer and incubated in the dark at 0 °C for 2 h. Excess sodium periodate was removed by incubation with 2 ml of 50% glycerol at 0 °C for 20 min. The incubation mixture was then dialyzed at 4 °C and dried. HPTLC resulted in a single band. The oxidized ganglioside was analyzed by NanoESI-MS/MS mass spectrometry.

MS/MS mass spectrometry. NanoESI-MS/MS-analysis was performed with a triple quadrupole instrument [VG micromass (Cheshire, UK) model Quattro II] equipped with a nano-electrospray source and gold-sputtered capillaries as described [17,18]. Parameters for cone voltage and collision energy of the different scan-modes are listed in Supplemental Table 1.

Ganglioside analysis. $3–20 \times 10^7$ cells were extracted successively with 5 ml each of methanol, chloroform–methanol (1:2, v/v), and chloroform–methanol (2:1, v/v) in an ultrasonic bath (5 min in ice cold water). The combined solvent extracts were evaporated at 25 °C under reduced pressure. The residue was homogenized in 5 ml of 0.88% aqueous KCl in an ultrasonic bath and desalted on a glass column filled with 600 mg Sep-Pak C18 coated 55–105 μm silica particles (Waters, Milford, MA, USA) as described [19]. The desalted lipids were separated on silica 60 HPTLC plates (Merck, Darmstadt, Germany), (solvent: chloroform–methanol–water (120:70:17, v/v/v), containing 0.02% (w/v) $CaCl_2$) and were immunostained as described [20,21]. Gangliosides were quantified by densitometry of the HPTLC plates with a Shimadzu PC 9000 scanner at 580 nm using orcinol staining and quantified standards.

Isolation of mouse liver mitochondria. Mitochondria were isolated from mouse liver (Balb/c, female, 6–8 weeks old, Charles River, l'Arbresle, France) by differential centrifugations and purified on Percoll gradient [22]. Protein concentration was determined using the micro-BCA assay (Pierce Chemical Company, Rockford, Illinois).

Monoclonal antibodies and preparation of GD3–7-specific antiserum. A culture supernatant of the GD3-specific monoclonal antibody (mAb) R24 (CD60a) was a kind gift from Johannes Müthing, Münster, Germany. For preparation of a GD3–7-specific antiserum, 2 Balb/c × C3H/F1 mice (Charles River, Sulzfeld, Germany) were immunized intraperitoneally with an emulsion of 50 μg purified GD3–7 in sterile physiological NaCl in complete Freunds adjuvant. After four injections in 3-week intervals, the serum was tested at a 1:100 dilution for GD3–7 and GD3 immunoreactivity using TLC immunostaining. The serum, named anti-GD3–7, reacted strongly with GD3–7 but not with GD3 and other non-oxidized gangliosides from human leukocytes.

Swelling and depolarization assays. For swelling and depolarization measurements, mitochondria (25 μg protein) were diluted in a hypo-osmotic buffer (10 mM Tris–MOPS, 5 mM succinate, 200 mM sucrose, 1 mM phosphate, 10 μM EGTA, and 2 μM rotenone, pH 7.4) and distributed into 96-well microtiter plates (200 μl/well). After addition of various doses of calcium (Ca^{2+}), carboxyatractyloside (CAT), GD3, GD3–7, and acGD3, the mitochondrial swelling was immediately measured by the decrease in absorbance at 540 nm for 1000 s. The depolarization of the mitochondria was measured concomitantly by the rhodamine 123 fluorescence dequenching assay (1 μM, λ_{exc}: 485 nm, λ_{em}: 535 nm, Molecular Probes). Both assays were adapted from [23,24] and were performed at 37 °C in a spectrofluorimeter (TECAN Genios, TECAN, Grödig, Austria).

PTPC purification and reconstitution into liposomes. The PTPC was purified from four rat brains (Wistar rats, male, 11–12 weeks old, Charles River) and reconstituted into cholesterol: phosphatidylcholine (1:50 w/w) liposomes [25,26]. The ability of GD3 and derivatives to activate the pore function of PTPC was evaluated by addition of alkaline phosphatase (which converts 4-methylumbelliferylphosphate (4-MUP) into the fluorochrome 4-methylumbelliferone) to measure the release of entrapped 4-MUP in comparison to calcium induced 4-MUP release [22]. The 100% of 4-MUP release was determined by adding 0.5 mM Ca^{2+} to proteoliposomes. The fraction enriched in the PTPC components, which was used for the reconstitution into liposomes was analyzed for the presence of ANT, voltage-dependent anion channel (VDAC) and cyclophilin D (CypD) by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 12%) and Western-blotting with a rabbit polyclonal serum against ANT (Genosphere, Paris, France), a rabbit polyclonal serum against VDAC (Genosphere, Paris, France), and a monoclonal antibody against CypD (Mitosciences, Eugene, Oregon).

ANT purification and reconstitution into liposomes. ANT was purified from rat heart mitochondria [22], checked to be VDAC and CypD-free, and reconstituted into proteoliposomes (phosphatidylcholin/cardiolipin (45:1, w/w)). Proteoliposomes were loaded either with 4-MUP in 10 mM KCl, 10 mM Hepes, 125 mM saccharose (pH 7.4), by sonication, washed on PD10 columns, dispensed in 96-well microtiter plates and incubated with the indicated agents at 20 °C. The release of 4-MUP was quantified by addition of alkaline phosphatase. The 100% of 4-MUP release was determined by adding 1 μM CAT to proteoliposomes. The fluorescence induced by the treatment of liposomes by another agent was calculated as a percentage of CAT-induced 4-MUP release.

Results

To test our hypothesis that the oxidation of GD3 is required for its pro-apoptotic function it was subjected to mild periodate oxidation and the effects of the oxidized and of the unchanged GD3 and of acGD3 on the PTPC and ANT function were compared. The purified oxidized GD3 was analyzed by nanoESI-MS/MS. Fragmentation of the oxidized GD3 revealed a characteristic fragment of m/z −519 instead of m/z −581, the latter being characteristic for the two linked sialic acids in GD3 (Fig. 1A and B). The difference represents a loss of 62 amu corresponding to $C_2H_6O_2$. This fits only with the 7-aldehyde but not the 8-aldehyde form of GD3. The loss of 62 amu is due to a breakdown of the terminal sialo-group as single-charged fragments corresponding to GM3, LacCer, GlcCer, and Cer are unchanged in comparison to corresponding GD3 spectra. For measurement samples were dissolved in methanol. Aldehydes easily form hemiacetals with alcohols. Hence, methanoyl-GD3–7 hemiacetals were also present and their fragmentation spectra revealed a fragment due to the loss of methanol (32/2 amu/e) but were otherwise very similar to GD3–7 fragmentation spectra (data are only shown for the species containing the Cer(d18:1, 22:0) lipid anchor, Fig. 1A–C).

The oxidation products of GD3 turned out to be exclusively GD3–7 as all ganglioside molecules detected with a general scan for sialic acids (m/z −87) were also identified with the fragment m/z −519, specific for GD3–7 and no signals corresponding to the molecular weights of educt GD3-species were found (Fig. 1D and E), also not with a scan for the two intact sialic acids (m/z −581) (data not shown). Scanning the oxidized GD3 sample with the neutral loss of 16 amu, the subset of methanoyl-hemiacetals was