

Different mechanisms are involved in apoptosis induced by melanoma gangliosides on human monocyte-derived dendritic cells

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Received on May 16, 2008; revised on February 4, 2009; accepted on February 6, 2009

Tumor escape is linked to multiple mechanisms, notably the liberation, by tumor cells, of soluble factors that inhibit the function of dendritic cells (DC). We have shown that melanoma gangliosides impair DC differentiation and induce their apoptosis. The present study was aimed to give insight into the mechanisms involved. DC apoptosis was independent of the catabolism of gangliosides since lactosylceramide did not induce cell death. Apoptosis induced by GM3 and GD3 gangliosides was not blocked by inhibitors of de novo ceramide biosynthesis, whereas the acid sphingomyelinase inhibitor desipramine only prevented apoptosis induced by GM3. Furthermore, our results suggest that DC apoptosis was triggered via caspase activation, and it was ROS dependent with GD3 ganglioside, suggesting that GM3 and GD3 induced apoptosis through different mechanisms.

Keywords: acid sphingomyelinase/apoptosis/ceramides/dendritic cells/gangliosides/melanoma

Introduction

Gangliosides are sialic acid-containing glycosphingolipids that modulate several cellular functions such as proliferation, cell adhesion, signal transduction, and apoptosis (Hakomori et al. 1998). It has long been known that the ganglioside composition is altered in tumor cells. For instance, while normal melanocytes only express GM3, melanoma cells overexpress a variety of gangliosides, the majority being GM3 and GD3 (Tsuchida et al. 1987). Furthermore, a number of tumors, including metastatic melanoma, shed gangliosides into their microenvironment (Portoukalian et al. 1978; Ladisch et al. 1983; Bernhard et al. 1989) and much evidence suggests that soluble gangliosides contribute to tumor-induced immunosuppression (McKallip et al. 1999). Recently, we have shown that GM3 and GD3

gangliosides purified from human melanoma tumors impair dendritic cell (DC) differentiation from human monocytes (Péguet-Navarro et al. 2003), as well as epidermal Langerhans cell maturation (Bennaceur et al. 2006), and induce apoptosis of both cell types. Indeed, ganglioside GD3 accumulation has been shown to be associated with Fas or ceramide-mediated apoptosis (De Maria et al. 1997) and requires ceramides produced by the action of acidic sphingomyelinase (De Maria et al. 1998). Metabolic products of gangliosides, like ceramides, have also been reported to induce apoptosis (Olshefski et al. 1996; Taha et al. 2006). In contrast, gangliosides have also been shown to protect L929 fibroblast cell line from TNF-mediated apoptosis (Koike et al. 1993). Furthermore, the induction of apoptosis in hematopoietic cells is regulated by the balance between apoptosis-inducing genes and -suppressing genes (McDonnell et al. 1996). However, the effect of gangliosides on the expression of these genes is unknown. Therefore, in the present investigation, we were interested in analyzing the mechanisms of GM3 and GD3 ganglioside-induced apoptosis by using different approaches.

Results

Purity of the ganglioside fractions

Thin-layer chromatography was carried out to assess the purity of the melanoma-derived ganglioside fractions. As shown in Figure 1, the GM3 and GD3 fractions were free of other lipids and no ninhydrin-positive contaminant could be detected on the thin-layer plate (not shown).

Dendritic cells in vitro take up and catabolize exogenous gangliosides

GD3 tritiated on the sphingoid base was added at a concentration of 50 $\mu\text{g}/\text{mL}$ to the culture medium at the onset of the dendritic cell differentiation process and, after 4 days incubation, the fate of GD3 was investigated. Lipid analysis found that about 30% of the cell-associated radioactivity was still in the form of intact GD3, with less than 2% migrating as GM3, and with less than 10% of the remaining radioactivity being present in sphingomyelin and ceramides (not shown).

Gangliosides induce apoptosis of DC

Figure 2 shows clearly that the presence of both GM3 and GD3 gangliosides in the culture medium of dendritic cells induces apoptosis. However, it should be pointed out that, although apoptosis was always observed under our experimental conditions, we experienced a fairly high variability, depending on the donors, in the yield of human dendritic cells and in their susceptibility to apoptosis by gangliosides during this process.

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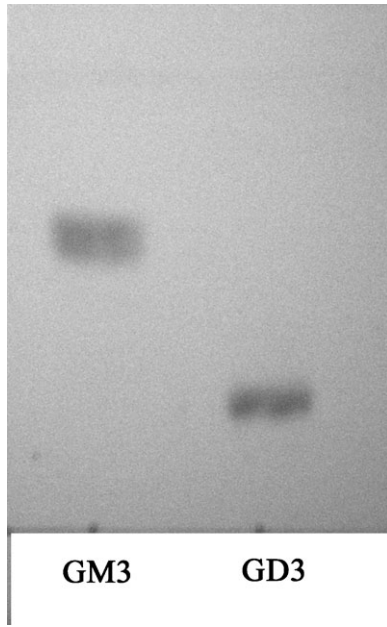


Fig. 1. Thin-layer chromatography of the GM3 and GD3 ganglioside fractions isolated by HPLC from the total melanoma gangliosides. Gangliosides were visualized using resorcinol-HCl reagent.

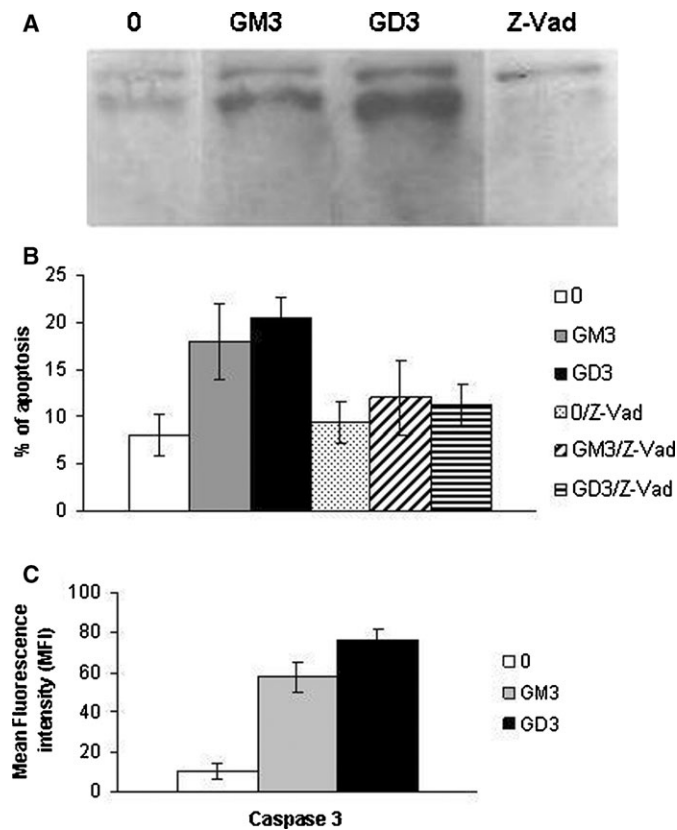


Fig. 2. Ganglioside-induced DC apoptosis is caspase dependent. To test whether a caspase-dependent pathway is involved in DC apoptosis, (A) PARP cleavage was detected. GM3- and GD3-induced apoptosis increased cleavage of PARP. (B) The apoptosis of DC was detected also with an Annexin/PI kit. The broad inhibitor of caspase Z-Vad was added every 2 days to the cell cultures both in the presence or absence of gangliosides. (C) GM3- and GD3-induced apoptosis increased caspase 3 activation.

Ganglioside-induced DC apoptosis is caspase dependent

To test whether a caspase-dependent pathway is involved in DC apoptosis, PARP cleavage was detected. PARP is involved in DNA repair and the cleavage of this enzyme, a target of caspase 3, has been used extensively as a marker of caspase 3 activity (Woo et al. 1998). The obtained results show that PARP is cleaved more in DC treated with gangliosides than untreated DC, in which the cleavage corresponds to spontaneous apoptosis (Figure 2A). On the other hand, apoptosis of DC was detected with an Annexin/PI kit. The broad inhibitor of caspase, Z-Vad, was added every 2 days to the cell cultures, both in the presence or absence of gangliosides. We observed that gangliosides do not induce DC apoptosis in the presence of Z-Vad (Figure 2B), and the apoptotic effects of GM3 and GD3 were abrogated. Furthermore, caspase 3 detection revealed that GM3 and GD3 gangliosides increase caspase 3 activation (Figure 2C).

GD3 but not GM3 ganglioside induced a loss of mitochondrial transmembrane potential

It has been suggested that mitochondria play an important role in cellular apoptosis (Gogvadze et al. 2008). To investigate the role of mitochondria in apoptosis induced by GM3 and GD3 gangliosides, the fluorescent JC-1 probe was used to detect any disruption of the mitochondrial transmembrane potential, as an early apoptotic event in DC cells treated or not by gangliosides. In apoptotic cells, the mitochondrial membrane potential was dissipated; thus, the JC-1 dye was dispersed in the cell as monomers detected in the FL1 channel. Representative dot plots and the percentages of apoptotic cells based on FL1-positive and FL2-negative signals are shown in Figure 3. The apoptotic cells are shown in the lower-right quadrant of the plots and it is clear that the presence of GD3 increases apoptosis by 15% whereas GM3 has no significant effect on apoptosis. The results thus showed that GD3 induced apoptosis via mitochondria, unlike GM3.

GD3 but not GM3 ganglioside induced production of superoxide anion

To explore the molecular mechanisms of ganglioside-induced apoptosis, we monitored superoxide anion production from DC treated or not by GM3 and GD3 gangliosides during 4 days by staining them with DHE, a fluorescence dye for superoxide anion, and subsequent flow cytometry analysis. In GM3-treated DC, superoxide anion production is not significantly increased as compared with controls. However, in the presence of GD3 ganglioside, DC superoxide anion production is increased (Figure 4).

Kinetic studies of ganglioside-induced apoptosis

Kinetic studies were carried out to determine the necessary time using melanoma-associated gangliosides GM3 and GD3 to induce DC apoptosis. In a previous study (Peguet-Navarro et al. 2003), we showed that upon addition of GM3 or GD3 on day 2 of the culture, apoptotic cells could be detected from day 4. In this study, GM3 and GD3 were added at the onset of cell culture, and DC were collected and washed after respectively 1, 2, and 6 days before measuring apoptosis by annexin/propidium iodide staining. When monocyte exposure to gangliosides was limited to the first or even second days of culture and apoptosis was measured the same day, the percentage of apoptosis in treated

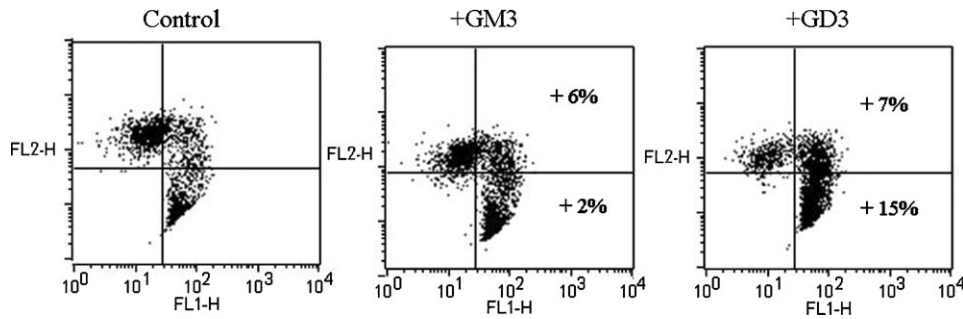


Fig. 3. GD3 ganglioside induced a loss of mitochondrial transmembrane potential. Gangliosides were added at the onset of culture, and cells were stained 6 days later with JC-1 dye reagent (FluoProbes). Cells were analyzed for apoptosis by flow cytometry (Becton Dickinson). In healthy cells, the JC-1 dye formed red fluorescent aggregates that were detected in the FL2 channel. In apoptotic cells, the dye was dispersed in the cell as green fluorescent monomers, detected in the FL1 channel. The dot plot of the X-axis (FL1), which is the log of JC-1 green fluorescent monomers, and the Y-axis (FL2), which depicts red fluorescent aggregates, was obtained by flow cytometry. The apoptotic cells (typically FL1-positive and FL2-negative) are indicated in the bottom-right quadrant.

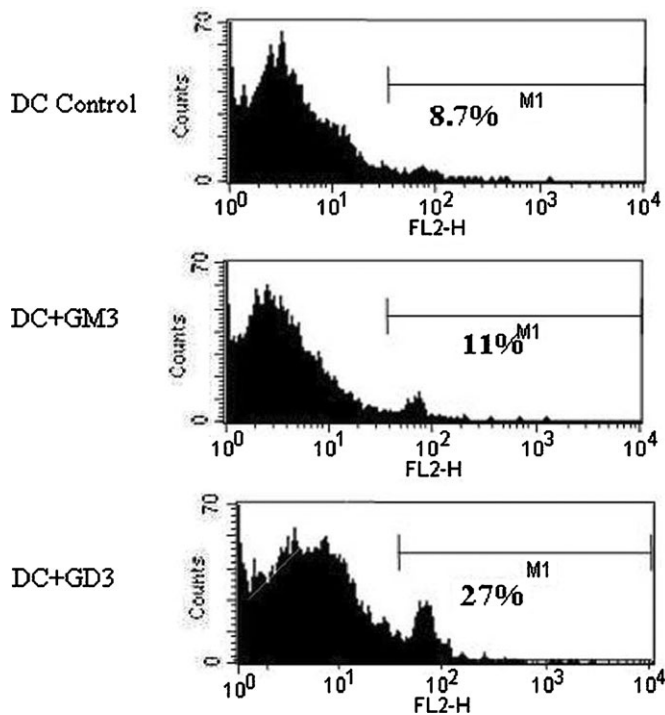


Fig. 4. Effect of GM3 and GD3 gangliosides on superoxide anion production during DC differentiation. Purified monocytes were cultured with GM-CSF and IL-4, in the presence or the absence of GM3 or GD3 (50 μ g/mL) gangliosides. Gangliosides were added at the onset of culture, and cells were stained 6 days later with dihydroethidium (DHE) staining and analyzed by flow cytometry.

DC was not significantly different from the untreated control cells. However, we observed on day 6 a high percentage of apoptotic cells on ganglioside-treated DC (Figure 5). This result suggests that several days are necessary for the apoptotic signal to be triggered following incubation with gangliosides.

Ceramides generated via the de novo pathway are not responsible for DC apoptosis

The addition of lactosylceramide (50 μ g/mL) derived from enzymatic hydrolysis of melanoma gangliosides to DC did not induce apoptosis, although its degradation pathway is the same

as that of GM3 and GD3 gangliosides, suggesting that cell death was not merely induced by products of the catabolism of gangliosides. Still, some data obtained from kinetic studies could suggest that ganglioside-induced DC apoptosis occurred through accumulation of endogenous ceramides. Therefore, to verify this possibility, de novo ceramide biosynthesis was blocked by the inhibitors fumonisin B1 (50 μ M) and/or myriocin (50 μ M), added at the beginning of cell culture in the presence or absence of gangliosides. The results obtained showed that FB1 and myriocin (data not shown), which are specific inhibitors of ceramide synthase, had no effect on ganglioside-induced apoptosis (Figure 6A), thus ruling out a ceramide-mediated mechanism of apoptosis.

GM3 ganglioside induces an accumulation of ceramides generated via the SMase pathway

Endogenous ceramide biosynthesis can also be generated by sphingomyelin hydrolysis by two kinds of sphingomyelinase: neutral and acid, which are inhibited respectively by glutathione (10 mM) and desipramine (1 μ M). These inhibitors were added with the presence or absence of gangliosides every 2 days during cell culture. Glutathione had no effect on DC survival (not shown) whereas desipramine decreased apoptosis triggered by GM3 ganglioside (Figure 6B).

aSMase mediates death of DC treated with GM3 but not with GD3 ganglioside

To investigate the involvement of aSMase in DC death, small interfering RNA (siRNA) technology was used. Introduction of siRNA oligonucleotide targeting *smpd-1/aSMase* mRNA reduced DC death treated by GM3 ganglioside but not with GD3 (Figure 7A). This result suggests that the activation of acid sphingomyelinase is responsible for DC apoptosis induced by GM3 ganglioside.

Effect of gangliosides on aSMase activity

For the determination of aSMase activity in vitro, we have developed a high-throughput method based on a fluorescent substrate analog (NBD-sphingomyelin) that is hydrolyzed by a cell lysate to form the corresponding ceramide. After the reaction, the remaining substrate and the product are extracted by organic solvent, separated by thin-layer chromatography and quantified by fluorescence using densitometer. Figure 7B shows that the

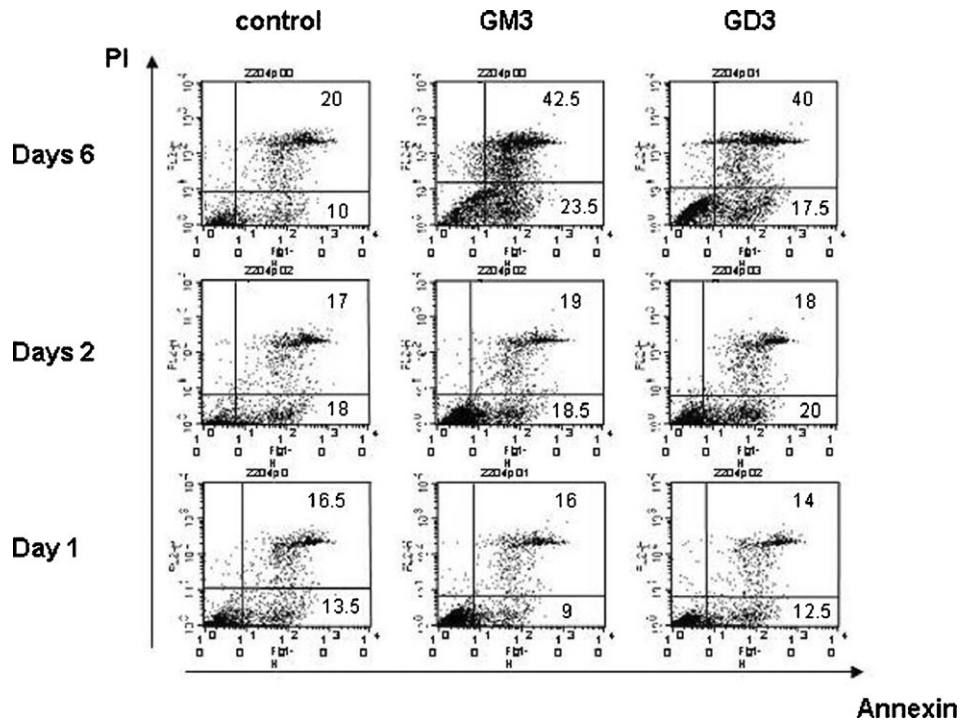


Fig. 5. Kinetic studies of ganglioside-induced apoptosis. GM3 and GD3 were added at the onset of cell culture, and DC were collected and washed after respectively 1, 2, and 6 days before measuring apoptosis by annexin/propidium iodide staining.

cleavage of NBD-sphingomyelin into ceramides by DC was enhanced by GM3, whereas GD3 had no effect.

Discussion

Gangliosides added exogenously to the culture medium induce alterations in several cellular events including cell growth, differentiation, maturation, and apoptosis, but the molecular events through which this occurs are largely unknown. Specific gangliosides such as GM3 and GD3 have been reported to induce DC apoptosis (Melchiorri et al. 2002; Peguet-Navarro et al. 2003; Bennaceur et al. 2006). In the 2003 study, it was observed that gangliosides induce apoptosis of DC during their differentiation from monocytes. The present study extends these results by investigating the mechanisms of apoptosis of DC induced by gangliosides.

In order to verify that the effects on dendritic cells reported in this study were indeed linked to the presence of the gangliosides, the following experiments were carried out as previously published (Bennaceur et al. 2006): aliquots of freshly purified ganglioside fractions were preincubated with specific antibodies for 1 h at 37°C with constant stirring, and then protein L-agarose (Sigma-Aldrich) was added for one more hour. After centrifugation, the solution was found to be depleted of gangliosides and none of the effects reported in our study could be seen using the depleted solution, thus strongly suggesting that the gangliosides are responsible for the observed effects on dendritic cells.

At the concentrations of gangliosides used in our study, these compounds seem to be only slowly catabolized, and we found that, after 4 days of incubation of dendritic cells with radioactive GD3, this ganglioside still accounted for 30% of the cell-

associated radioactivity whereas metabolites such as ceramides and sphingomyelin accounted each for less than 10%. These results are within the same range of percentages as those previously reported by groups investigating the fate of exogenous gangliosides on other types of human cells such as fibroblasts (Chigorno et al. 1997).

Several studies have shown that ceramide production is increased in apoptotic cells before morphological modifications, thus suggesting that ceramides are involved in the transduction of the signal triggering cell apoptosis (Mathias et al. 1998). In fact, it was demonstrated that the supernatant of melanoma tumors induced ceramide biosynthesis and apoptosis in bone marrow-derived DC (Kanto et al. 2001). Moreover, it is known that proliferating tumors shed gangliosides in their microenvironment. In the previous study, kinetic studies showed that ganglioside-induced apoptosis was detected between 4 and 6 days after their addition to the DC. Such a delay suggests a cellular accumulation of substances mediating apoptosis.

Different approaches were used to clarify the involvement of ceramides in DC apoptosis, firstly by blocking ceramide synthesis with specific inhibitors and secondly by treating the cells with inhibitors of ceramide catabolism. Initially, we demonstrated that GM3- and GD3-induced apoptosis was caspase dependent since it could be reversed by Z-Vad, a specific inhibitor. This result was confirmed by the strong cleavage of PARP on DC treated with gangliosides. We also measured directly caspase 3 activity in DC treated by GM3 and GD3 gangliosides, and the results suggested that both gangliosides increased caspase 3 activity. Furthermore, it had been shown that GD3 ganglioside induces an apoptotic signal via CD95 receptor and activates caspases (De Maria et al. 1997). On the other hand, our results regarding the mitochondrial inner-transmembrane

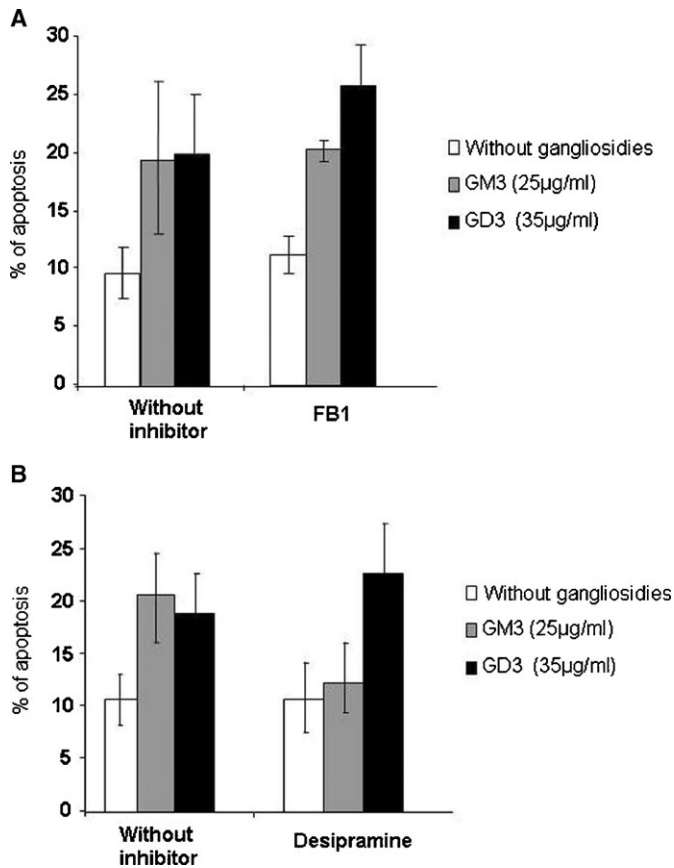


Fig. 6. Ceramides generated via the de novo pathway are not responsible for DC apoptosis. **(A)** The de novo ceramides biosynthesis was blocked with Fumonisin B1 (50 µg/mL). These inhibitors were added at the beginning of cell culture in the presence or absence of gangliosides. **(B)** GM3 ganglioside induced an accumulation of ceramides via the acid SMase pathway. Acid sphingomyelinase inhibited by desipramine (1 µM) was added in the presence or absence of gangliosides every 2 days during cell culture. Results are representative of four independent experiments.

potential ($\Delta\Psi_m$), as determined by the JC-1 fluorescence shift, demonstrated that GD3, but not GM3, induced a loss of mitochondrial transmembrane potential. Recent studies indicate that mitochondria exhibit functional and structural changes that regulate apoptosis (Hüttemann et al. 2007). Measurements of $\Delta\Psi_m$ revealed that cells undergoing apoptosis have a significantly lower $\Delta\Psi_m$ than normal cells. These results were confirmed after ROS detection by DHE staining. In GM3-treated DC, superoxide anion production was not significantly increased when compared with controls. However, GD3 ganglioside increased superoxide anion production. These results suggest that unlike GM3, GD3 ganglioside is involved in DC apoptosis via a direct effect on mitochondria. In the other approach to understand the induced DC apoptosis mechanism caused by GM3 and GD3 gangliosides, lactosylceramide derived from melanoma gangliosides by sialidase treatment was used. Since the addition of lactosylceramide had no effect on DC (data not shown), it is highly unlikely that the observed effects could be triggered by ceramides produced by the cleavage of added gangliosides because lactosylceramide is a metabolite of gangliosides that is further degraded into ceramides during the catabolic process. Free ceramides are released mainly through the SMase pathway

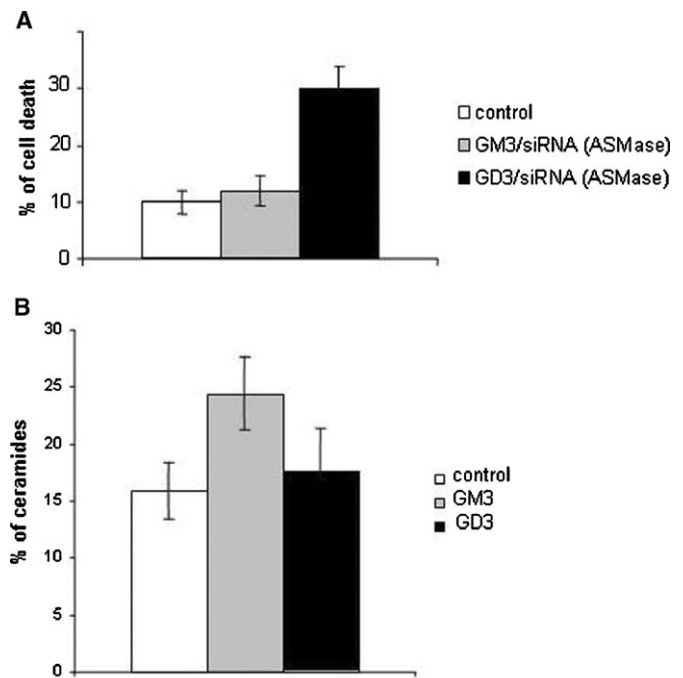


Fig. 7. **(A)** aSMase RNAi-mediated downregulation of apoptosis induced by GM3 but not with GD3 gangliosides. **(B)** DC treated by GM3 but not with GD3 gangliosides increased ceramides production via the acid sphingomyelinase pathway.

or the de novo biosynthesis pathway. The enzymes involved in these pathways reside in different intracellular compartments, leading to different localizations of ceramides (Mathias et al. 1998). Furthermore, in the apoptotic process, cells may respond differently to some forms of stress and stimulus by generating ceramides from different pathways. Indeed, the induction of de novo biosynthesis of ceramides during apoptosis is due to stress stimulus such as cytotoxic drugs on T lymphocytes (Boland and Foster 1997), ionizing radiations on skin cells (Farrell et al. 1998) or exogenous fatty acids on hematopoietic cells (Paumen et al. 1997). In our study, apoptosis induced by GM3 and GD3 was not reversed by the addition of fumonisin B1, suggesting that ceramides generated via the de novo pathway are not responsible for DC apoptosis. On the other hand, the implication of the SMase pathway in the apoptotic process seems to be generally better recognized. Several studies showed apoptosis on different kinds of cells following stimuli such as TNF- α , Fas, interleukin-1, deprivation of growth factors (Mathias et al. 1998), cytotoxic drugs (Bezombes et al. 2001), and ultra-violet light (Chatterjee and Wu 2001). We have seen that glutathione and trimipramine (not shown), which are inhibitors of neutral sphingomyelinase, did not reverse the apoptosis induced by gangliosides. By contrast, desipramine which blocks acid sphingomyelinase activity completely prevented apoptosis induced by GM3, whereas it had no effect on GD3-induced apoptosis. These results were confirmed since DC treated by GM3, but not with GD3 gangliosides, increased ceramide production via the acid sphingomyelinase pathway. Equally, aSMase iRNA-mediated downregulation of apoptosis was detected after treatment with GM3, but not with GD3. Although GM3 is also a breakdown product of GD3 ganglioside, the failure of the latter ganglioside to

influence the aSMase pathway could be explained by the fact that membrane-bound sialidase cleaves GD3 directly into lactosylceramide without detectable intermediate GM3, as observed by Merritt et al. (1987). In summary, our results suggest that ganglioside GM3 induces the production of ceramides via SMase which accounts for the apoptosis of DC, whereas GD3 induces apoptosis via a direct action on mitochondria, thus showing that GM3 and GD3 induce apoptosis of DC through different mechanisms.

In conclusion, we have now unveiled some of the biochemical pathways leading to apoptosis of dendritic cells when they are incubated with melanoma-derived GM3 and GD3 gangliosides, as described in our first report (Peguet-Navarro et al. 2003). However, the mechanisms accounting for the impairment of the major functions of dendritic cells such as allogeneic T-cell proliferation and migration to lymph nodes (Bennaceur et al. 2006) remain to be investigated.

Materials and methods

Purification of monocytes

Mononuclear cells were obtained from peripheral blood of healthy donors by centrifugation on Lymphoprep. Monocytes were purified by negative magnetic depletion using hapten-conjugated anti-CD3, CD7, CD19, CD45RA, and CD56 (MACS, Miltenyi Biotec, Bergisch, Germany) and a magnetic cell separator (Midi MACS), according to the manufacturer's instructions. The technique resulted routinely in >85% purity, as assessed by anti-CD14 staining and flow cytometry analysis.

Western blotting for PARP detection

To isolate the whole cell lysates for immunoblotting, the cell pellets were suspended in the lysis buffer. The samples were incubated at 4°C for 30 min, and then centrifuged at $14,000 \times g$ for 15 min. The supernatants were collected, and total protein was quantified. Fifty micrograms of lysate was separated by 12% SDS-PAGE and transferred to the polyvinylidene difluoride membrane blocked with defatted milk at 5% in PBS (w/v). Immunostaining was performed with rabbit polyclonal primary Abs specific for PARP (Sigma-Aldrich) followed by incubation with goat polyclonal anti-rabbit IgG conjugated to HRP (Sigma-Aldrich).

Caspase 3 assay

The cells were stained with an anti-caspase 3 antibody as a primary antibody, and then with goat anti-mouse IgG coupled with fluorescein isothiocyanate as a secondary antibody. Fluorescence analysis was performed on 10^4 cells using a flow cytometer (Becton Dickinson, Mountain View, CA) and the Cell Quest software.

Reagents and enzymatic inhibitors

The cell permeable inhibitor of caspase-1 and caspase-3, *N*-benzyloxycarbonyl-Val-Ala-Asp (O-Me) fluoromethyl ketone (Z-VAD-fmk), diphenylene iodonium (DPI), L-cycloserine, myriocin, fumonisins B1 (FB1), glutathione (GSH), desipramine were obtained from Sigma-Aldrich, France.

Purification of gangliosides

Gangliosides were purified from human melanoma tumors in our laboratory. GM3 and GD3 were isolated by high-performance liquid chromatography on a 250-4 Si100 column (Merck, Darmstadt, Germany) with a Hitachi L-6200 apparatus (Hialeah, FL) using a ternary gradient of hexane-isopropanol-water (55/36/9 to 55/30/15, v/v/v) at a flow rate of 0.25 mL/min. Fractions of 0.5 mL were collected, and elution was monitored by thin-layer chromatography on HPTLC silica gel 60 plates (Merck) migrated in chloroform/methanol/0.2% aqueous calcium chloride (60/35/8, v/v/v). The plates were visualized by heating at 150°C after spraying with a resorcinol-HCl reagent. The GM3- and GD3-containing fractions were separately run a second time to ensure optimal purity. Aliquots of both gangliosides were tritiated on the long-chain base (Schwarzmann 1978) using sodium borotritide (American Radiolabelled Chemicals, St. Louis, MO).

Production of dendritic cells

The culture medium was RPMI 1640 supplemented with L-glutamine (Life Technologies, Grand Island, NY), 1% gentamicin (Sigma-Aldrich, St. Louis, MO), and 10% heat-inactivated FCS (Myoclon; Life Technologies), hereafter called the complete medium. Purified monocytes (10^6 cells/mL) were cultured for 6 days in 12-well tissue culture plates (Costar, Cambridge, MA) in the complete medium supplemented with recombinant human GM-CSF (200 ng/mL, Schering-Plough) and IL-4 (33 ng/mL, Schering-Plough), in the presence or absence of melanoma-derived gangliosides, respectively, GM3 and GD3 (50 µg/mL). On days 2 and 4, cells were fed with fresh medium and cytokines. Enzymatic inhibitors were added at different times to the culture.

Apoptosis detection by annexin/propidium iodide

Apoptosis is characterized by exposure of phosphatidylserine residues on the cell surface. DC apoptosis was measured using the annexin V-FITC kit, with dead cells identified by propidium iodide (Immunotech). For this assay, 5000 events were collected on a FACScan II cytometer and analyzed with CellQuest software.

Mitochondrial apoptosis assay

To investigate whether GM3 and GD3 gangliosides induce apoptosis via mitochondria, we used the FluoroProbes JC-1 mitochondrial apoptosis detection reagent (Interchim, Montluçon, France) as per the manufacturer's instructions. This assay is based on the disruption of the mitochondrial inner-membrane electrochemical potential using a fluorescent dye. Briefly, during DC differentiation GM3 and GD3 gangliosides are added into cells culture on days 2 and after 4 days of culture, mitochondrial apoptosis assay was performed with JC-1 reagent, and then analyzed immediately by flow cytometry. In healthy cells, JC-1 dye accumulated in the mitochondria as aggregates and was detected in the FL2 channel. In apoptotic cells, the dye was dispersed throughout the cell as monomers and detected in the FL1 channel.

Detection of ROS production

Dihydroethidium staining (DHE 5 µM) was used to evaluate intracellular levels of superoxide (O_2^-) in DC treated or not by

gangliosides. DHE is freely permeable to cells and in the presence of superoxide is oxidized to fluorescent ethidium bromide which gets trapped inside the cells by intercalation into DNA. After gangliosides treatment, DC were washed three times with PBS. Next, DHE (5 μ M) was added into each well of six-well plates (Falcon, Franklin Lakes, NJ) and incubated for 20 min at room temperature. Staining was stopped by washing cells three times with PBS, and then analyzed by FACS Calibur (Becton Dickinson, Mountain View, CA).

Fluorescence assay of acid sphingomyelinase (aSMase)

For determination of aSMase activity in human DC as a function of substrate concentration, human DC (2×10^6) were incubated with 10 nmol N-C12:0-NBD-sphingomyelin (Matreya, Pleasant Gap, PA) in a total volume of 200 μ L for 1 h at 37°C. The reaction was stopped by adding 2 mL CHCl_3 - CH_3OH , 2:1 (v/v) for lipid extraction. The solvent of the organic phase was evaporated under N_2 , the residue was redissolved in 100 μ L CHCl_3 , and the resultant solution was applied onto a TLC plate. After migration in a solvent system chloroform-methanol-water 65:25:4 (by volume), the plate was scanned with a CS-930 Chromatoscan densitometer (Shimadzu, Kyoto, Japan) equipped with a fluorescence apparatus. The resulting ratio NBD-ceramide/NBD-sphingomyelin allowed the calculation of aSMase activity.

siRNA-mediated gene silencing

To suppress aSMase mRNA expression, siRNA specific for this enzyme (sc-41650 from Santa Cruz Biotechnology, Paris, France) was used. The efficiency of siRNA treatment on acid SMase activity was checked as detailed above, and the activity was found to be decreased by at least 85%. Oligonucleotide (2 nmoles) targeting aSMase/*smpd1* was transfected into cells with QiagenTM Lipid Reagent and cultured for 48 h. At the end of the incubation period, DC death was assessed with propidium iodide.

Funding

A bursary from the Matrix Association (Laboratory of Dermatological Research, EA 41-69, Building R, Edouard Herriot Hospital, 69437 Lyon Cx 03, France). Funding to pay the Open Access publication charges for this article was provided by the Matrix Association.

Conflict of interest statement

None declared.

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