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Apoptosis of human breast carcinoma cells in the presence of disialosyl gangliosides: II. Treatment of SKBR3 cells with GD3 and GD1b gangliosides

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Apoptosis, or programmed cell death, plays an important role in many physiological and diseased conditions. Induction of apoptosis in cancer cells has been monitored during the cells' progression to apoptosis by anti-cancer drugs and inhibitors of the cell surface glycolipids, gangliosides and SA-Le^x biosyntheses [Basu, S (1991) *Glycobiology*, 1, 469–475; and *ibid*, 427–435] in animal tissues and human carcinoma cells, respectively. Induction of apoptosis in cancer cells by cell surface glycolipids in the human breast cancer (SKBR3) cells is the aim in this study. We have employed the disialosyl gangliosides (GD3 and GD1b) to initiate apoptosis in SKBR3 cells grown in culture in the presence of ^{14}C-L-Serine. At lower concentrations (0–20 μM) of exogenously added non-radioactive GD3, GD1b, or bovine ganglioside mixture (GM1:GD1a:GD1b:GT1a 2:4:4:2), the incorporation of radioactivity in both ^{14}C-sphingolipid and ^{14}C-ceramide was higher. However, at higher concentrations (20–100 μM), wherein apoptosis occurred in high frequency, the ^{14}C-incorporation decreased in both GSLs and ceramide. Apoptosis induction was monitored by the concomitant appearance of caspase-3 activation and the binding of a fluorescent dye PSS-380 to the outer leaflet of phosphatidyl-serine. These results indicated that, in addition to many unknown cell surface glycoconjugates GD3 or GD1b (disialosyl ganglioside) could play an important role in the regulation of breast carcinoma cell death.
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Keywords: **apoptosis, breast carcinoma cells, caspase-3, SKBR3, ceramide, gangliosides, GD3, GD1a, GD1b, PSS-380, propidium iodide, phosphatidyl-serine**

Introduction

Apoptosis plays an important role in developmental morphogenesis, cancer biology and disease pathology. It is a phenomenon of physiological cell death and an essential part of the cell turnover. It was recognized over 100 years ago [1]. More recently it is in the limelight with the discovery of death receptors and their ligands [2]. During the last two decades, different apoptotic signaling pathways have been discovered and various components of their machinery have been identified [3].

Phospholipids (PL) and glycosphingolipids (GSLs) are believed to play an important role for the stress-responses of many eukaryotic cells. A relationship between lipid mediators and apoptosis has been established in recent years [4]. Different families of glycosphingolipids (ganglio-, globo- and lacto-) [5], including short and long-chain gangliosides [6] have been proved to take part in the cell apoptotic processes [7]. The amphipathic nature of gangliosides consists of a hydrophobic ceramide moiety, that anchors in the outer phospholipid bilayers of the plasma membranes, whereas the hydrophilic oligosaccharide moiety is extended outside the cell surface [8]. These oligosaccharides may contain neutral sugars with inner cores (lacto-; Galβ 1-4Glc- or gangliotetraose-; Galβ1-3GalNAcβ1-4Galβ1-4Glc-). To these inner cores, one or more sialic acid (NeuAc or NeuGc) is attached (e.g. GD3 or GD1b) (Figure 1).

Biosynthesis *in vitro* of both GD3 [9] and GD1b [10,11] has been established in embryonic tissues (Figure 2) and hybrid-GSLs in cancer cells (Figure 3) [11–13]. The glycosyltransferases that catalyze the synthesis of these disialosyl-gangliosides are expressed in the Golgi apparatus [14,15].

The metastatic invasive properties of the tumors cells have been correlated with the acidic-GSLs of the lacto-family (e.g. sialosyl-Le^x, SA-Le^x or SA-Le^a) [16–19]. Biosynthesis *in vitro* of these carcino-embryonic cell surface antigens (SA-Le^x/

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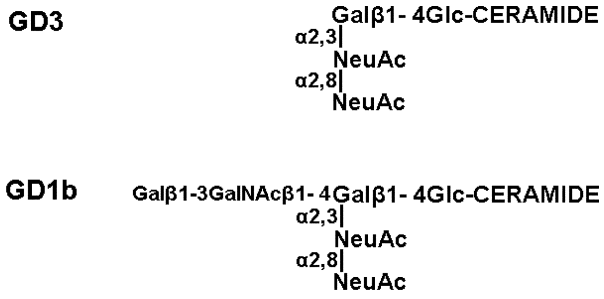

Figure 1. Structures of Disialosyl Gangliosides, GD3 and GD1b.

SA-Le[a]) has also been established in recent years [20–27]. GD3 occurs in the CNS and optic nerve [28]. Otherwise it is a minor ganglioside in the normal tissue [29]. However, it has been detected as a major GSL in meningiomas [30], gliomas [31], breast cancer cells [32], melanoma [33] and colorectal carcinomas [34]. Increased GD3 concentration during neural differentiation and growth rate of CHO-K1 cells [35] has been reported recently. GD3 also sensitizes human hepatoma cells to cancer therapy [36]. Expression of GD3 gangliosides on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms [37]. However, chimeric anti-GD3 monoclonal antibody by KM871 is proved to enhance *in vitro* antibody-dependent cellular cytotoxicity [38] and inhibition of the proliferation of human malignant glioma cells *in vitro* [39]. GD3 ganglioside has been recognized in recent years as an apoptotic agent in oligodendrocytes [40] and neuronal cells in culture [41]. Comprehensive review articles have been published to examine the mechanism by which GD3 can regulate cell proliferation or apoptosis [42,43]. However, very little investigation has been done to study the effect of other disialosyl-gangliosides on apoptosis of normal or cancer cells.

In this study we report for the first time the initiation of apoptosis in breast carcinoma SKBR3 cells in the presence of both GD3 and GD1b (Disialosyl-gangliosides).

Material and methods

Materials

SKBR3 breast cancer cell line was a gift from Dr. Sipra Banerjee of Cleveland Clinic, Cleveland, OH. Cell culture medium powder DMEM was from Gibco-BRL/Invitrogen Corporation (Carlsbad, CA). Penicillin, streptomycin, and L-glutamine were from Gibco-BRL. Fetal bovine serum was purchased from Intergen (Purchase, NY) and Gibco. ^{14}C-L-Serine was from Moravek Biochemicals (Brea, CA). Human ganglioside GD3 and ganglioside mixture (GM1:GD1a:GD1b:GT1a = 2:4:4:2) were prepared previously in our laboratory. GD3 (isolated from milk) was a gift sample from Dr. Goro Hanagata (Japan) and the Fluorescent dye PSS-380 [44] was a gift from Dr. Bradley Smith of the Department of Chemistry and Biochemistry at the University of Notre Dame. Whatman GF/A glass filters were from Fisher Scientific (Pittsburgh, PA). Pierce BCA Micro Protein Assay kit was from Pierce Biotechnology, Inc. (Rockford, IL). Rabbit anti-caspase3 polyclonal antibody was from BioMol Research Lab, Inc. (Plymouth Meeting, PA). Goat anti-rabbit IgG antibody-ALP (alkaline phosphatase) conjugate, NBT (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) ALP developing dye, and all other regular reagents were from Sigma (St. Louis).

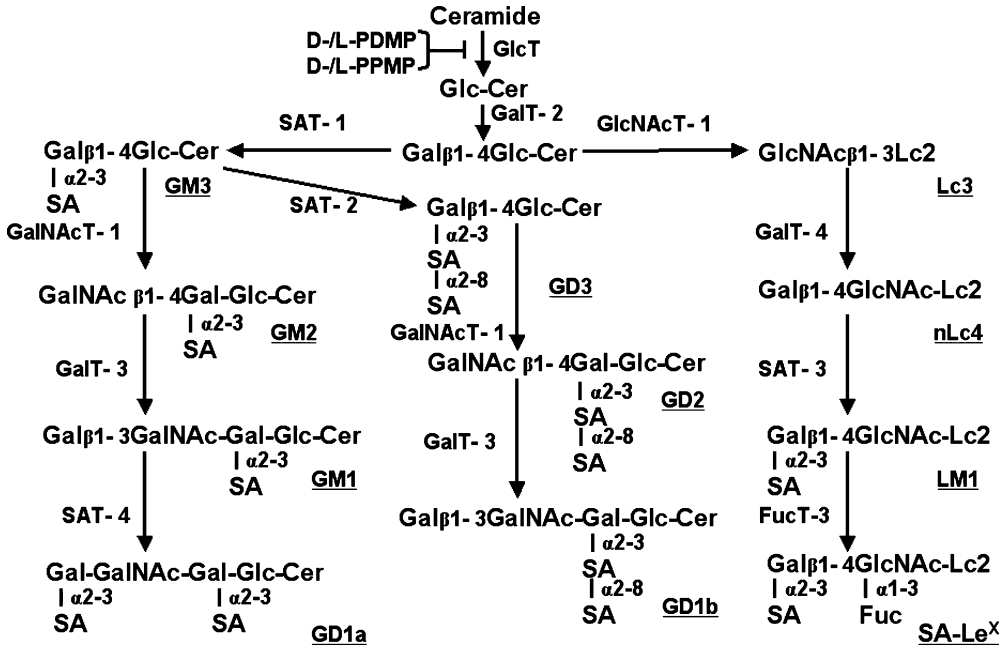

Figure 2. Proposed pathways for biosynthesis of mono-, di-sialosyl glycosphingolipids and sialosyl Lewis X (SA-Le^X).

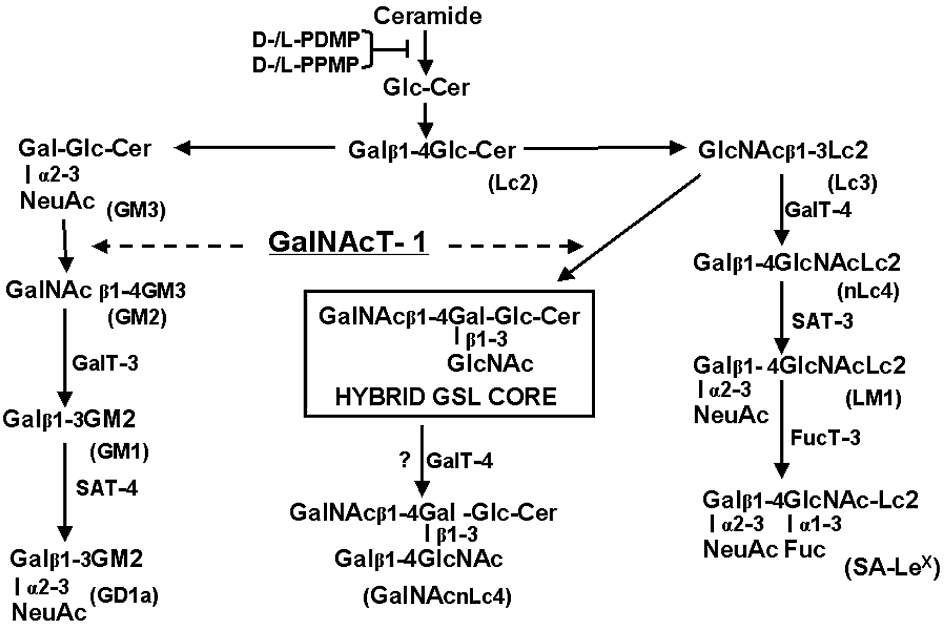

Figure 3. Proposed pathways for biosynthesis of hybrid-GSLs (Guinea pig bone marrow/ Embryonic Chieken Brain).

Cell culture

Cultures of human breast carcinoma SKBR3 cells were grown [45] in 50-ml (25-cm^2) Falcon plastic T-flasks containing 5 ml of Dulbecco's Modified Eagle Media (D-MEM), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin, and 50 mM L-glutamine. Incubation was carried out in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. When the monolayer cells reached 90% confluence, the cells were subcultured with 0.25% trypsin digestion or synchronized 2 times (24 h each) with 0.5 mM hydroxyurea in the culturing medium before treatment of apoptotic reagents. The glycosphingolipid and disialosylganglioside treatments were performed after the synchronization of confluent SKBR3 cells at different dose or time conditions in the presence of radioactive ^{14}C-L-Serine (0.5 μCi/5 ml/T-flask). After treatment, the pictures of control or apoptotic cells were taken with the Polaroid® 667 b/w films with the optical microscope (10×). The cells in each T-flask were harvested and washed 2 times with PBS before resuspension in 5 ml PBS. The densities of live and dead cells in the suspension were measured by cell counting with Trypan Blue staining method.

Incorporation of radioactivity by GF/A filtering

0.5 ml suspension of cells were loaded onto a GF/A glass filter, which had been treated with 50 mM sodium pyrophosphate, the procedure published recently [46]. Then the sample on each GF/A disc was washed twice with 5% TCA followed by 2 times with a chloroform/methanol (2:1) wash plus 2 times with the acetone wash, or 2 times acetone wash only. Each cell sample was repeated twice. After that, the GF/A discs were fully dried and counted in a toluene scintillation solution.

Distribution of radioactivity in phospholipids and glycosphingolipids

The cells from 1 ml suspension were resuspended in 200 microliter 0.1 M NaOH plus 500-microliter chloroform: methanol (2:1) and incubated at 37°C for 1 h. After that, the cell lysate was centrifuged at 3000 rpm, 4°C for 10 min. Both upper layer and lower layer (50 microliter each) were spotted on the 4 cm^2 Whatman-3MM paper and followed by scintillation-counting on a Beckman counter. Incorporation of radioactivity was quantitated by TLC as described before [46].

Western blot for identification of activation of caspases

Cells (0.5 ml aliquots) were pelleted and resuspended with 100 ml lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol) followed by homogenization with 3 × 10 sec sonication. The protein concentrations were measured by Micro-BCA assay (Pierce). Then the homogenized samples were incubated at 37°C for 1 h before 5 min of denaturation at 95°C and being loaded onto SDS-PAGE gel. The protein mixture (20 micrograms) was loaded for each sample and electroblotted to nitrocellulose membranes. Nonspecific binding was blocked by incubation in Tris-buffered saline containing 5% bovine serum albumin [47] and 0.1% Tween-20 for 1 h at room temperature. The blots were then incubated overnight at 4°C in blocking buffer containing the primary antibody. Antibodies used were a rabbit polyclonal anti-caspase-3 antibody raised against full-length human caspase-3 diluted 1:1,000. Afterward, membranes were washed and incubated with anti-rabbit IgG-Alkaline phosphatase conjugate (1:3,000; Sigma). Antibody—alkaline phosphadase activity was visualized using the

NBT-BCIP reagent in the AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$).

Fluorescence staining of PSS-380 and propidium iodine

Cells cultured on Falcon Microslide System (Fisher) were synchronized 2 times (24 h each) with 0.5 mM hydroxyurea before treatment with apoptotic reagents at different conditions. After that, the cells were washed 2 times with TES buffer (5 mM N-tris[Hydroxymethyl]-2-aminoethanesulfonic acid: TES, 150 mM NaCl, pH 7.4), then incubated with 200 microliter new TES buffer containing 25 μM PSS-380 and 0.25 μg/ml propidium iodide at 37°C for 10 min. The buffer was removed after staining, and the cells were washed with TES buffer once before observation for fluorescence.

Results

1. Incorporation of ^{14}C-serine in radioactive sphingolipids in the presence of disialosylgangliosides

Synchronized human breast carcinoma SKBR3 cells were treated with varying concentrations of disialosyl gangliosides GD3 (Figure 4) (10–50 μM) or with a ganglioside mixture including GD1b (Figure 4) (10–100 μM) in the presence of uniformly labeled L-^{14}C-Serine. The apoptotic morphological changes of the cells were observed in 48 h of treatment (Data are not shown). Then the incorporation of radioactivity was studied with GF/A filtering assay (to analyze the incorporation of L-^{14}C-Serine into sphingolipid plus phosphatidylserine) or alkaline-chloroform/methanol extraction assay (to analyze the incorporation of L-^{14}C-Serine to sphingolipid only). The methodology of both assays was discussed in our paper published in the 1st issue of the series in this journal [46]. The CPM values versus live or total cell numbers (quantitated per 10^6 cells) are shown in Figure 4a–d. Both GF/A filtering and alkaline-chloroform/methanol extraction curves of GD3-treated cells showed the same pattern of incorporation of L-^{14}C-Serine (Figure 4a and b). GD3 increased the total lipid (sphingolipid plus phosphatidylserine) or sphingolipid synthesis at low concentrations (about 10–20 μM) and inhibited lipid synthesis at high concentrations (about 20–50 μM). A ganglioside mixture can clearly decrease the total sphingolipid biosynthesis (Figure 4c) as well as it induces apoptosis in SKBR3 cells (Figure 7) at higher concentrations (80 μM).

In live cells, low concentrations of the ganglioside mixture slightly increased the incorporation of L-^{14}C-Serine to sphingolipid cells. The data of incorporation of L-^{14}C-Serine into protein (radioactivities obtained after chloroform/methanol wash in GF/A filtering assay), which are not shown in this paper, indicated the same trend of inhibition by GD3 or ganglioside mixtures. This means during the apoptosis induced by GD3 and ganglioside mixture (GM1:GD1a:GD1b:GT1a 2:4:4:2), the changes in total sphingolipids (sphingomyelin plus glycolipids) are similar

2. Detection of translocation of membrane phosphatidylserine using a novel fluorescent dye

One important phenomenon of apoptotic cells is the randomization of the distribution of phosphatidylserine (PS) between the inner and outer leaflets of the plasma membrane. In normal cells, the phosphatidylserine is present in the inner leaflet of cell membrane [48]. During apoptosis, phosphatidylserine-flip to the outer leaflet of the cell membrane can be detected (Figure 5). Recently, our collaborator in the same department at Notre Dame proposed a new synthetic dye PSS-380, which can bind phosphate derivatives with negative charges (for example: phosphatidylserine or DNA at the physiological pH) [44]. But normal cells without membrane damage are not permeable to PSS-380. PSS-380 could be used as membrane phosphatidylserine detector in the early stage of apoptosis (Figure 5). In the later stage of apoptosis, the cell membrane permeability changes, then both PSS-380 and propidium iodide (a DNA binding dye) can enter to the cell nucleus. In this experiment, synchronized SKBR3 cells were treated at first with disialosyl gangliosides, washed twice with TES buffer (5 mM TES, 150 mM NaCl, pH 7.4) and then the dyes PSS-380 (25 μM) and propidium iodide (0.25 μg/ml) were added.

Within in 6 h of treatment of SKBR3 cells with GD3 (Figure 6) and GD1b (Figure 7), the dye PSS-380 [44] binds to the outer leaflet phosphatidyl-serine (PS) without any apparent damage (Figures 6 and 7). The degree of apoptosis increased with increasing concentration of the disialosyl-gangliosides. However, after 24 h of the onset of apoptosis with GD3, the nuclear membrane and DNA damage were evidenced by the appearance of bright red fluorescence (Figure 8). The propidium iodide dye bound to nuclear DNA with red fluorescence.

3. Western blot analysis of caspase-3 activation in apoptotic carcinoma cells by disialosyl gangliosides

Caspase-3 is the effector caspase in the apoptosis signal transduction pathway (Figure 9). It is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins [49–56]. When activated it cleaves proteins by recognizing the amino acid sequence DEVD. Upon recognition of the target proteins, the nucleus is broken down, starting with the disassembly of the chromosomes. The human inactive form of caspase-3 (pro-caspase-3) is a 32 kDa protein. Processing of pro-caspase-3 to the activated form, in apoptotic cells, is detectable primarily as loss of intensity from the 32 kDa band and appearance of lower molecular mass subunits 20 kDa and 17 kDa. We studied extensively (Figure 10) the Caspase-3 activation in SKBR3 cells in the presence of GD3 or ganglioside mixture. The activation profiles of Caspase-3 as evidenced in SKBR3 cells in the presence of GD3 (10–50 μM) and ganglioside mixture (5–25 μM) are shown on Figure 10. The activation of caspase-3 showed dose-dependent increase with GD3 or GD1b (present in the ganglioside mixture).

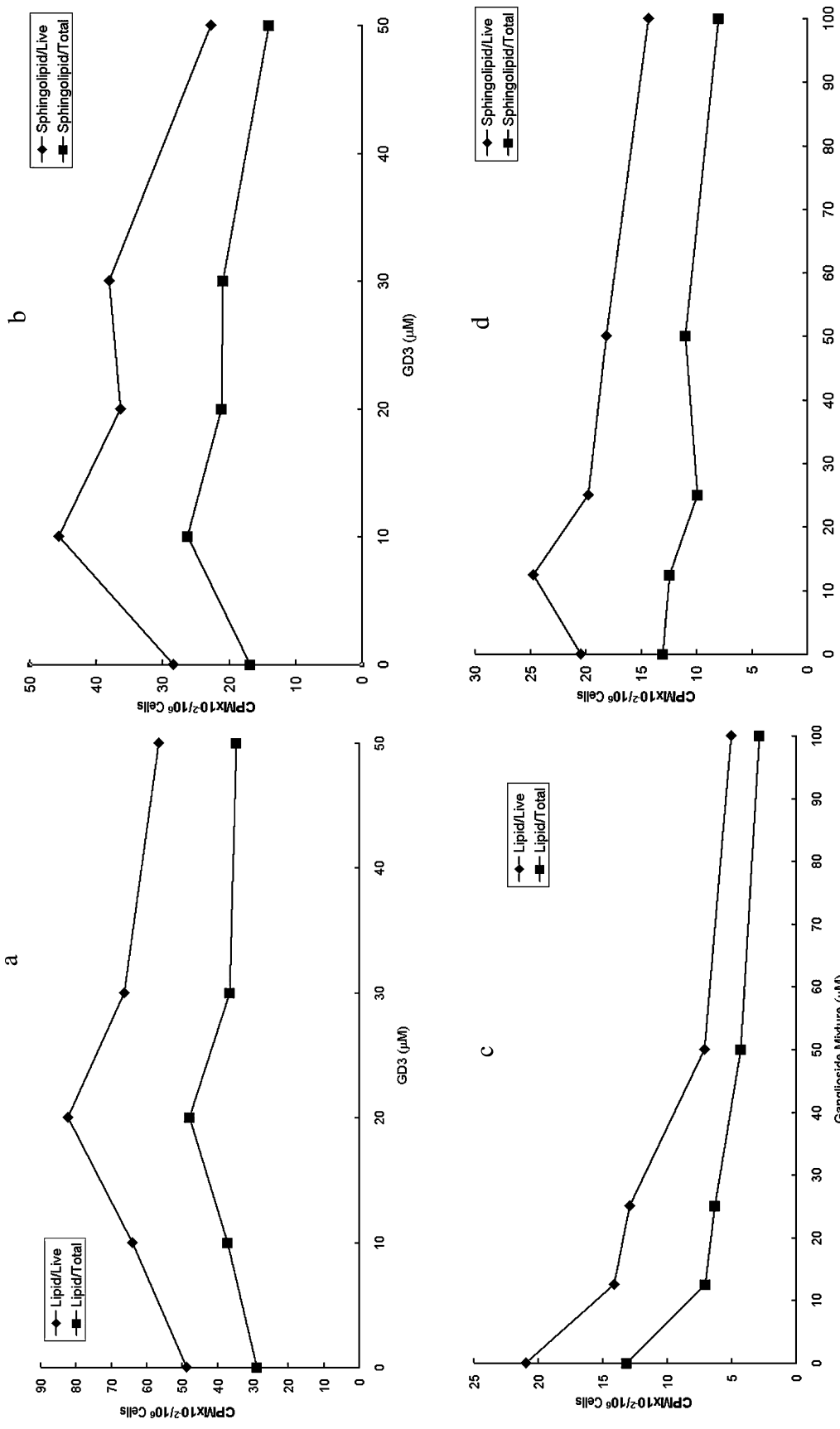

Figure 4. Effect of disialosyl gangliosides on lipid and sphingolipid biosynthesis of SKBR3 cells. (a) Effect of GD3 on total lipid biosynthesis; (b) Effect of GD3 on sphingolipid biosynthesis; (c) Effect of gangliosides mixture (GM1:GD1a:GD1b:GT1a = 2:4:4:2) on total sphingolipid biosynthesis; (d) Effect of gangliosides mixture (GM1:GD1a:GD1b:GT1a = 2:4:4:2) on sphingolipid biosynthesis. Each point represents average of the numbers generated from 4 independent determinations.

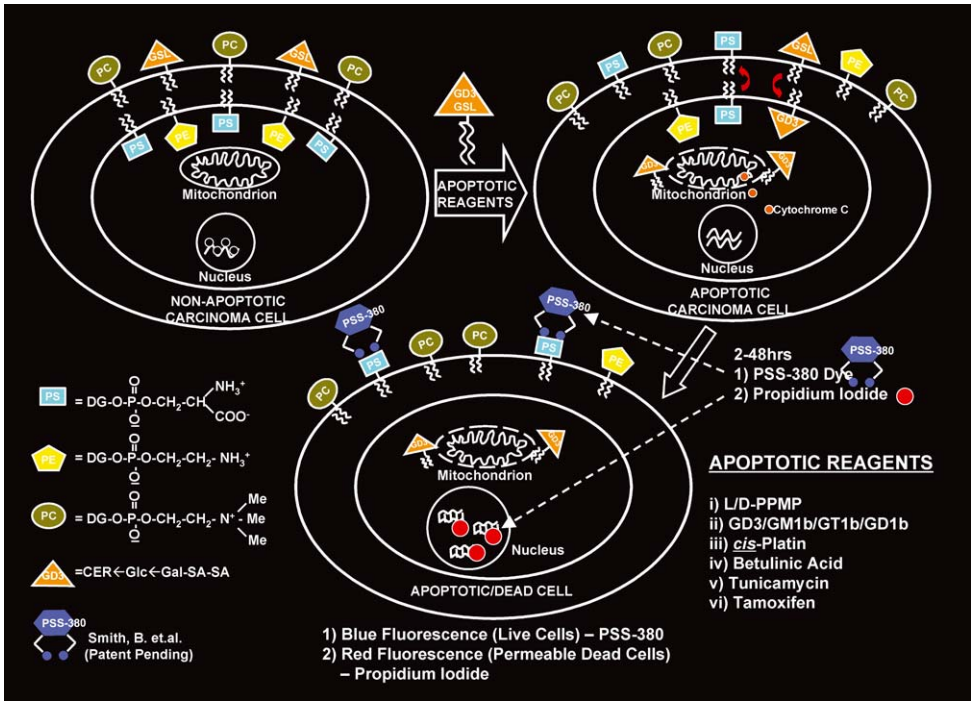

Figure 5. Identification of apoptotic cancer cells using fluorescent dyes: PSS-380 and Propidium iodide.

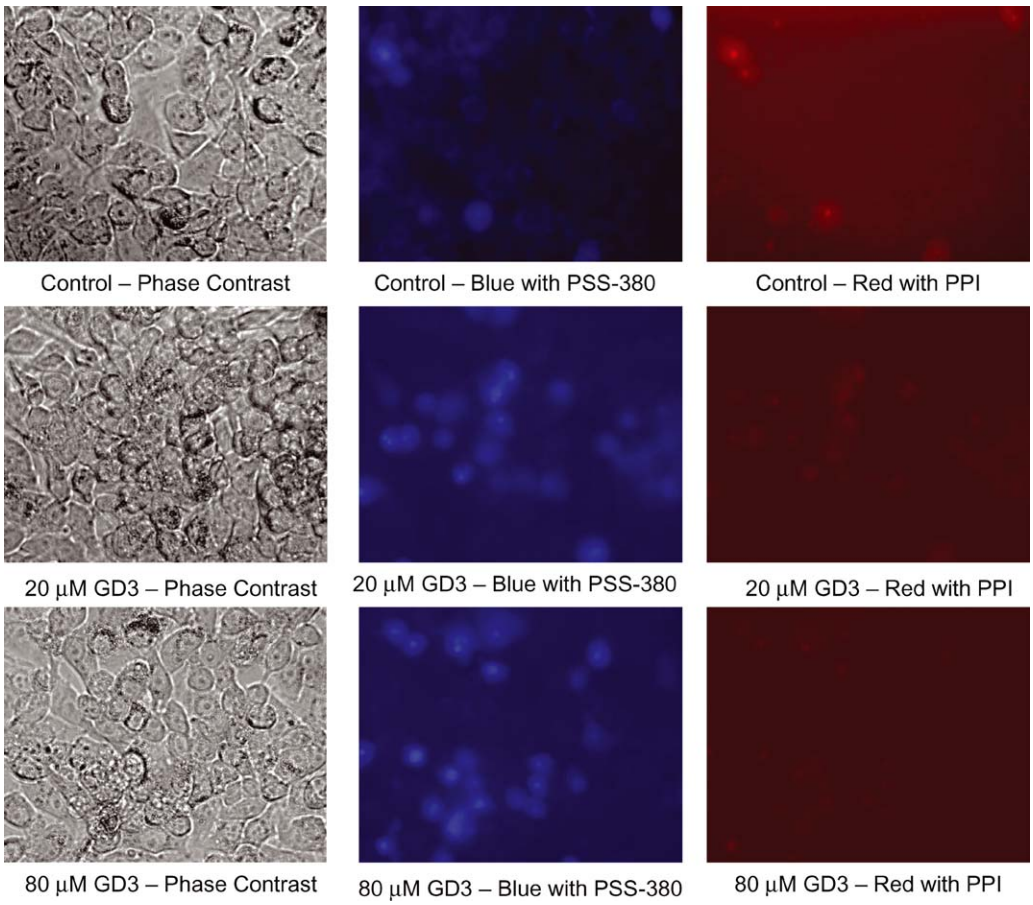

Figure 6. Apoptosis of breast cancer carcinoma (SKBR3) cells stained with PSS-380 and propidium iodide (Effect of GD3/6 h).

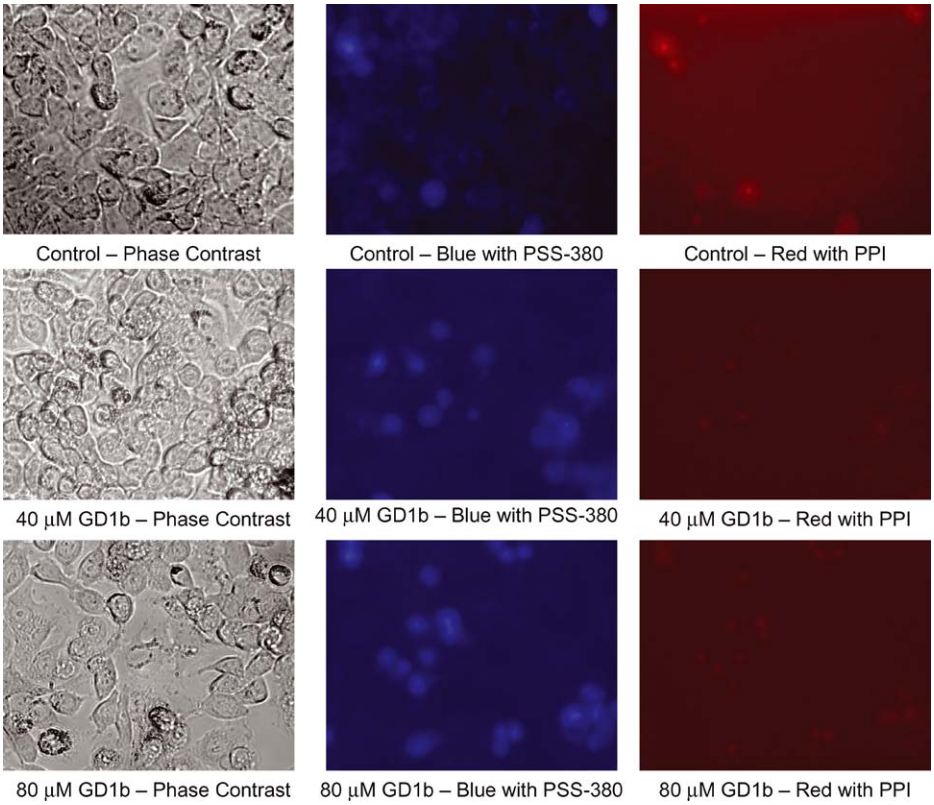

Figure 7. Apoptosis of breast cancer carcinoma (SKBR3) cells stained with PSS-380 and propidium iodide (Effect of GD1b/6 h).

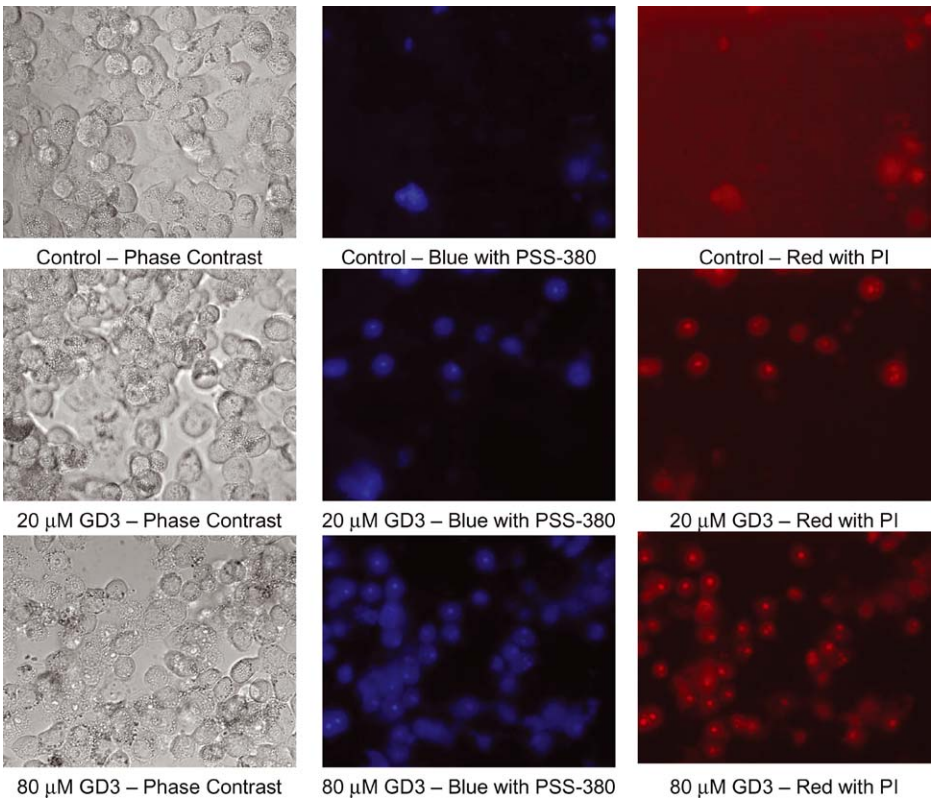

Figure 8. Apoptosis of breast cancer carcinoma (SKBR3) cells stained with PSS-380 and propidium iodide (Effect of GD3/24 h).

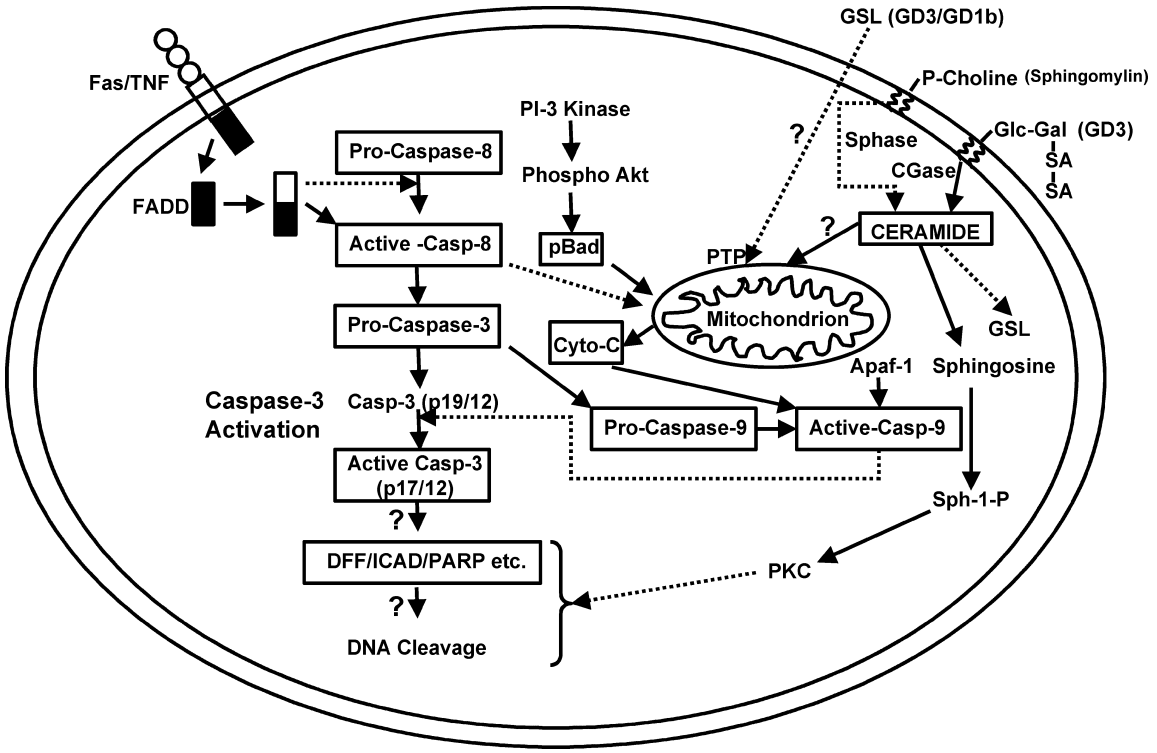

Figure 9. Caspase-3 activation cascade during apoptosis by disialosyl gangliosides.

Discussion

At the cellular level, it is suggested that apoptosis can be programmed by expression of some specific genes whose function is to cause cell death [50,55]. The notion is emerging that the aging process is under genetic control. Some of the genes known to play an important role in this process are part of the signal-transduction pathway, in which caspase-3 is activated [49,58]. Until now very little study has been published on the role played by ganglioside structures in the activation of caspase-3 and cell death. Several reports [34,40–43] are available on the relation of GD3-induced apoptotic process. Recent studies reported on the attachment of GD3 on the mitochondrial membrane [59–61] and the role of ceramide in the opening of the mitochondrial permeability transition pore [52,57–61]. It has been proved recently that GD3 recruits reactive oxygen species to induce cell proliferation and apoptosis in human aortic smooth muscle cells [62]. Immuno-histochemical studies with the colon tissue from the Farber-diseased patients showed co-existence of GD3 and the activated caspase-3 with K-18 (or 18 kDa) peptide in the cell bodies [63]. However, the mechanism of GD3 entry to the cells and its role in the generation of increased cellular concentration of ceramide [64] is not clear at present. Our present studies suggest that the disialosyl group (NeuAcα2-8NeuAcα2-3-) attached to lactosylceramide (Galβ1-4Glc-Cer), in GD3 as well as gangliotetraosyl ceramide (Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer) in GD1b is perhaps important for the transport of oligoglycosyl-ceramide across the membrane of live carcinoma cells. It is a time-dependent process with concomitant induction of apoptosis in these cells. We measured PSS-380 binding to the outer phosphatidylserine molecules by the fluorescence studies (Figures 5–7) and binding of propidium iodide to the damaged DNA molecules in the nuclei of the highly apoptotic cells.

In this study, we examined the effect of two disialosylgangliosides (GD3 and GD1b) on the incorporation of ^{14}C-L-Serine in the total sphingolipids (^{14}C-labeled sphingomyelin, ^{14}C-labeled glycosphingolipids). SKBR3 cells incubated between 10–20 μM of GD3 or GD1b showed maximum incorporation. Increase of ^{14}C-sphingolipid might be due to equilibration of *de novo* biosynthesis of radioactive ceramide and nonradioactive ceramide generated from the hydrolysis of GD3 or GD1b as it entered in the cells. The pool of nonradioactive ceramide during this incubation time (6–48 h) is not known. However, free ^{14}C-ceramide has not been detected in the radioactive sphingolipid pool (alkali stable). On the otherhand during L-PPMP induced apoptosis of Colo-205 cells free ^{14}C-ceramide was characterized [46]. However, with the progression of apoptosis, the incorporation of ^{14}C-Serine was reduced.

Translocation of phosphatidyl-serine from inside to the outer leaflet (Figure 5) was evident with the appearance of blue fluorescence within 6 h (Figures 6 and 7). At first, the blue fluorescence was observed only on the surfaces (plasma membrane) of the cells, but with the progression of apoptosis the fluorescence of the nuclear membrane also appeared. The heavy fluorescence at the center of each cell was visible. Cells were treated with PSS-380 dye (for binding of phosphatidylserine)

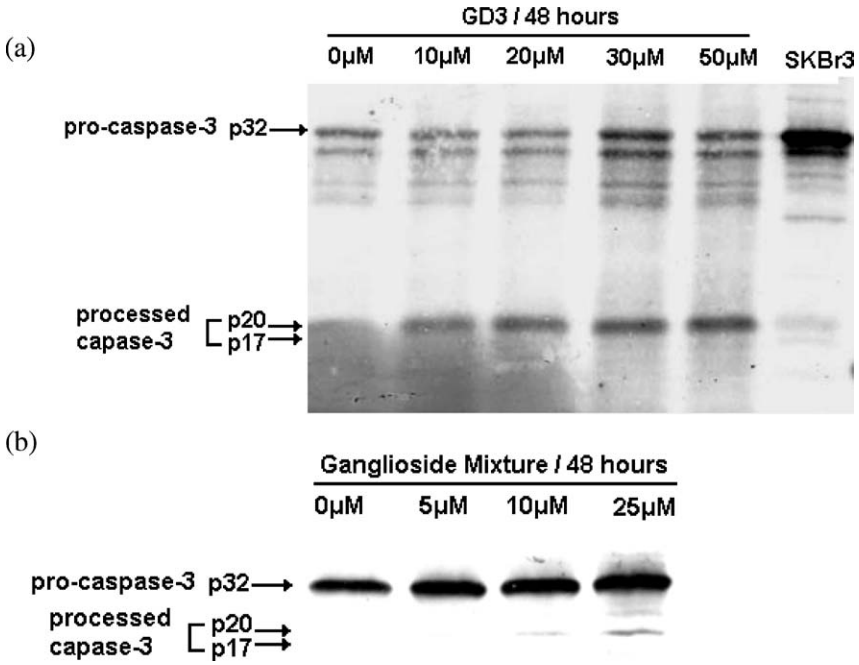

Figure 10. Activation of caspase-3 during apoptosis induced by GD3, GD1b and bovine brain ganglioside mixture (GM1:GD1a:GD1b:GT1a = 2:4:4:2) in SKBR3 cells.

and propidium iodide (for binding of DNA). In 6 h, very little red fluorescence was observed (Figures 6 and 7), proving that in 6 h after treatment of these cells with GD3 or GD1b in these cells the apoptotic process is initiated but the membrane is not yet damaged. However, after 24 h of incubation, the outer plasma membrane and the inner nuclear membrane are damaged (Figure 8) with the progress of apoptosis in the presence of GD3 (shown in Figure 8) and GD1b [65].

The Western blot results with anti-caspase-3 PAb obtained with SKBR3 cells (see Method section) give rise to the hypothesis that both GD3 and GD1b initiate the caspase-3 activation cascade (Figure 9) to enhance the cell death process, enhanced by further degradation of DNA. The cells treated between 24 h and 48 h showed DNA degradation as evidenced by DNA laddering experiments (data not shown; results will be published elsewhere).

It has been known for the last four decades that human cells display different patterns of cell surface GSLs from normal or untransformed cells [17,66]. Overexpression of specific disialosyl- gangliosides (GD3, GD2, GD1b and GQ1b) has been observed on several tumor cell surfaces such as melanoma [67], neuroblastoma [68], lymphoma [7], breast-[32], prostate [69], ovarian-[70] and colon carcinoma cells [70–72]. On the other hand colon cancer cell surface GSLs are mostly of the lacto-family, such as sialyl-Lewis X and sialyl-Lewis A type (NeuAcα2-3Galβ1-3(Fucα1-3)-GlcNAcβ1-3Galβ1-4Glc-Cer) [73]. As mentioned before, stepwise biosyntheses of these glycosphingolipids have already been established (Figures 2 and 3) [11–15,20–27]. Shedding of cell surface GSLs is a characteristic of cancer cells [29]. However, the underlying mechanism by which the released GSLs evoke any biological effect such as apoptosis is not fully understood as yet. GSLs of the ganglio-family (containing inner core GalNAcβ1-4Galβ1-4Glc-Cer) can be toxic to those tumor cells containing lacto-family (containing inner core GlcNAcβ1-3Galβ1-4Glc-Cer). Ceramide can be generated in the normal or cancer cells by L-PPMP, an inhibitor of Glc-Cer biosynthesis [72,73], or it could be generated by rapid degradation of sphingomyelin (Figure 11) by acidic or neutral sphingomyelinase [74–78]. Recently, the ceramide glycanase that cleaves between ceramide and the oligosaccharides has been reported from clam [79], rat mammary tissues [80–82] and breast cancer cells [83]. The ceramide glycanase activity could also generate a rapid increase of ceramide concentration (Figure 11) in mammary tissues or in cancer cells.

It is interesting to note that GD3 had been isolated from bovine cream-milk [84] and buttermilk [85,86]. In this apoptosis study we also used milk GD3. It is known that milk contains

1. **Ceramide-generated from inhibition of the first committed step in the biosynthesis of glycosphingolipids (L/D-PPMP; L/D-PDMP in Colo-205/SKBr3)**
2. **Ceramide-generated from breakdown of glycosphingolipids.(GD3 & GD1b Gangliosides in SKBr3)**
3. **Ceramide-generated from breakdown of sphingomyelin.**

Figure 11. Metabolic steps provoking apoptosis.

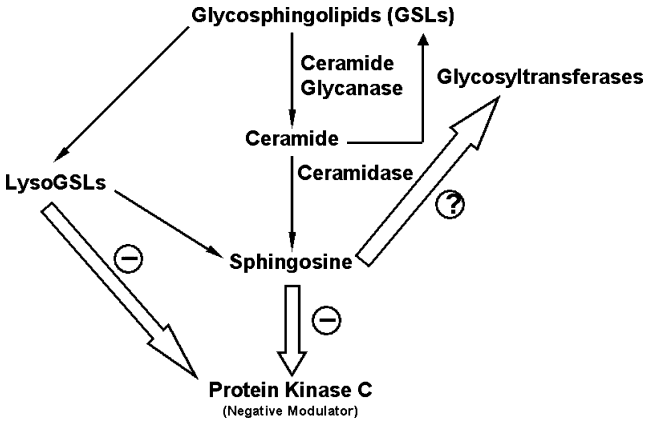

Figure 12. Effect of sphingosine as a biomodulator.

plenty of broken mammary cells and tissues. The question remains whether GD3 or any diasialosyl-ganglioside causes apoptosis of normal breast cells. Our present *in vitro* study of apoptosis of human breast cancer cells in the presence of disialosyl-gangliosides is important for further investigation of the role of these gangliosides (GD3 or GD1b) in: (i) signal transduction, (ii) initiation of caspase-cascade activation, (iii) initiation of breast cancer or (iv) normal cell death during milk secretion. If ceramide moieties of these GSLs are involved in this process, they could regulate the signal transduction pathway by binding of sphingosine-1-PO_3 [87] to its various receptors [88,89] or directly affecting the protein kinase C modulation [90] as depicted in Figure 12.

It is possible that agents such as GD3 or L-PPMP [80] that cause apoptosis of breast and colon carcinoma cells can be important for cancer chemotherapy.

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