GD3 Recruits Reactive Oxygen Species to Induce Cell Proliferation and Apoptosis in Human Aortic Smooth Muscle Cells*

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Sialic acid containing glycosphingolipids (gangliosides) are expressed on the surface of all mammalian cells and have been implicated in regulating various biological phenomena; however, the detailed signaling mechanisms involved in this process are not known. We report here a novel aspect of disialoganglioside, GD3-mediated regulation of cell proliferation and cell death via the recruitment of reactive oxygen species (ROS). A low concentration (2.5–10 μM) of GD3, incubated with human aortic smooth muscle cells for a short period of time (10–30 min), stimulates superoxide generation via the activation of both NADPH oxidase and NADH oxidase activity. This leads to downstream signaling leading to cell proliferation and apoptosis. However, [3H]GD3 incubated with the cells under such conditions was found in a trypsin-sensitive fraction that was separable from endogenous GD3. The exact mechanism causing ROS generation and downstream signaling remains to be elucidated. The uptake of GD3 was accompanied by a 2.5-fold stimulation in the activity of mitogen-activated protein (MAP) kinase and 5-fold stimulation in cell proliferation. Preincubation of cells with membrane-permeable antioxidants, pyrrolidine dithiocarbamate, and N-acetylcysteine abrogated the superoxide generation and cell proliferation. In contrast, at higher concentrations (50–200 μM) GD3 inhibited the generation of superoxides but markedly stimulated the generation of nitric oxide (NO) (10-fold compared with control). This in turn stimulated mitochondrial cytochrome c release and intrachromosomal DNA fragmentation, which lead to apoptosis. In sum, at a low concentration, GD3 recruits superoxides to activate p44 MAPK and stimulates cell proliferation. In contrast, at high concentrations GD3 recruits nitric oxide to scavenge superoxide radicals that triggered signaling events that led to apoptosis. These observations might have relevance in regard to the potential role of GD3 in aortic smooth muscle cell proliferation and apoptosis that may contribute to plaque rupture in atherosclerosis.

Glycosphingolipids (GSL) are ubiquitously expressed components of mammalian plasma membranes and have been implicated in the control of cell growth regulation through modulation of transmembrane signaling (1–3). Gangliosides are a species of GSL that contain sialic acids. GD3 is a disialoganglioside implicated in cell growth and proliferation. GD3 is overexpressed in some tumors, such as human melanoma in which it serves as a tumor antigen (4–8). Increased levels of GD3 have also been associated with proliferative diseases like atherosclerosis (9, 10). Recently, it was reported that the endogenous expression of GD3 synthase leads to proliferation of PC12 cells through the Ras-MAPK pathway (11). On the other hand, the overexpression of GD3 synthase leads to an increased level of GD3 that in turn contributes to apoptosis in human leukemic cells (12). In addition, studies suggest that GD3 mediates TNF-α and Fas/Apo-1/CD95-induced apoptosis in such cells. Collectively, these studies imply that GD3 might have a dual role in modulating cell proliferation and apoptosis. However, the signaling mechanisms involved in this phenomenon have not been evaluated. We rationalized that because cultured human aortic smooth muscle cells and normal human aorta contain a significant amount of GD3, it may well be implicated in proliferation. On the other hand a marked increase in the level of GD3 in human plaque from patients with atherosclerosis may contribute to plaque instability via inducing apoptosis.

Reactive oxygen species (ROS) are usually generated in response to diverse external stimuli, such as TNF-α, TGF-β, PDGF, EGF (13), and lipid second messengers, e.g. lysosphosphatic acid (14) and lactosylceramide (15). At low concentrations ROS may play the role of an intracellular messenger of various molecular events (16), including cell proliferation and apoptosis. However, the generation of large amounts of ROS is considered cytotoxic and contributes to apoptosis. ROS represents multiple molecular species, including singlet oxygen, superoxides (O2-), H2O2, NO, peroxynitrite (ONOO-), the thiperoxyl radical (RSOO2•), and the hydroxyl radical (HO•). Superoxides are the early molecular species of ROS that are generated as a consequence of the interaction of cells with external stimuli that in turn generate H2O2, HO•, etc. The generation of ROS has been related to the activation of transcriptional factors, for example, AP-1, NFκB, and mitogen-activated protein kinase (p44 MAPK) implicated in cell proliferation. On the other hand, NO as well as the generation of high levels of O2•− activate caspases that in turn lead to the dissolution of the cell membrane and nuclear components resulting in apoptosis. Collectively, these studies and our own
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(15–17) led us to hypothesize that GD3 may well recruit ROS to induce cell proliferation and apoptosis.

In this paper, we report that exogenously supplied \[^{3}H\]GD3 associates with the cell membrane mainly in a trypsin-sensitive state. Subsequently, it recruits ROS’s such as O\(_2^–\) and NO as biological sensors that serve a dual role in cell proliferation and apoptosis, respectively, in aortic smooth muscle cells (A-SMC). Because GD3 is a major GSL in normal A-SMC, and its level is markedly increased in human aortic plaque intima (8), our findings may be relevant to the pathological sequelae including the progression of atherosclerosis via A-SMC proliferation and the apoptosis of A-SMC that may contribute to plaque rupture.

MATERIALS AND METHODS

Isotopes and Chemicals—\[^{3}H\]Thymidine and \[^{32}P\]ATP (222 TBq/ mmol) were purchased from Amersham Biosciences. Bovine milk GD3 was purchased from Matreya (Pleasant Gap, PA) and labeled with \(^3\)H, employing palladium-catalyzed reduction of the sphingosine double bond (18). \[^{3}H\]GD3 was purified further by chromatography in silica gel Lichro prep S60 (E. Merck, AG Darmstadt, Germany) with chloroform/methanol/water (60:40:9, v/v). The specific activity of the \[^{3}H\]GD3 was 147.9 MBq/\(\mu\)g. A stock solution of GD3 was prepared in dimethyl sulfoxide and added to the culture medium to achieve the final desired concentrations of gangliosides. The maximum concentration of GD3 was added to the cells (10 \(\mu\)M) and attained a peak level at about 10 min of incubation with GD3 and determined by (15) using 250 \(\mu\)g lucigenin as the electron acceptor and either 100 \(\mu\)M NADPH or 100 \(\mu\)M NADH as an electron donor. In some experiments, NADPH oxidase activity was measured in the membrane preparations in the presence of 1 mM rotenone (mitochondrial poison).

The protein content was measured by the Lowry et al. (21) method with bovine serum albumin as the standard.

Immunoprecipitation and MAP Kinase Activity Assay—A-SMC was lysed in 100 \(\mu\)l of radioimmuno precipitation buffer containing 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 10 mM sodium fluoride, 1 mM Na\(_2\)VO\(_4\), 1 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin, 25 mM Tris/HCl, pH 7.4, 1% Triton X-100, and Nonidet P-40. The lysate was centrifuged and immunoprecipitated with anti-MAP kinase antibody conjugated with agarose as described earlier (22). Immunocomplex was directly used for MAP kinase activity assay (22). Briefly, 25 \(\mu\)l of total reaction mixture contained 1 mg/ml myelin basic protein peptide (APRTFGGR), 50 \(\mu\)M \[^{32}P\]ATP (1,800 cpm/\(\mu\)mol), 0.5 mM adenosine 3'5'-cyclic monophosphate-dependent protein kinase inhibitor, and assay dilution buffer containing 30 mM \(\beta\)-glycerophosphate, 20 mM MOPS, pH 7.2, 20 mM MgCl\(_2\), 5 mM EGTA, 1 mM dithiothreitol, 0.5 mM Na\(_2\)VO\(_4\), and 2-3 \(\mu\)g of immunoprecipitated protein. The reaction was initiated upon the addition of \[^{32}P\]ATP for 15 min at 30 °C and terminated with the addition of 10 \(\mu\)l of ice-cold 40% trichloroacetic acid. One part of the reaction mixture was run into 15% SDS-PAGE, and the gel was dried and autoradiographed.

Internucleosomal DNA Fragmentation Assay—Cells were treated with various agonists and antagonists (12, 14) and the DNA was isolated and analyzed by DNA electrophoresis as described earlier (23).

Cytochrome c Release Assay—Following incubation with agonists, cells were washed, harvested in ice-cold phosphate-buffered saline, and suspended in 100 \(\mu\)l of extracting buffer containing 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl\(_2\), 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 0.25 mM succrose (24). Cytosol was prepared and the level of cytosolic cytochrome c was determined by Western blotting using a monoclonal anti-cytochrome c antibody.

Measurement of Nitric Oxide Production—Nitrite production, a stable product of NO, was measured by the Griess reagent as described previously (25). The cells were incubated with the agonist and antagonists. At the end of incubation, 500 \(\mu\)l of culture medium was mixed with an equal volume of Griess reagent (1 part of 1% sulfanilamide in 2.5% phosphoric acid and 1 part of 0.1% naphthylethlenediamine dihydrochloride). The colorimetric absorbance at 550 nm was measured, and the nitrite concentration was determined using a standard curve generated using sodium nitrite.

RESULTS

Time Kinetics of \[^{3}H\]GD3 Incorporation into Human Aortic Smooth Muscle Cells—As shown in Fig. 1 (top panel), total incorporation of \[^{3}H\]GD3 into human aortic smooth muscle cells occurred very rapidly during the 30-min duration of this study. However, very little radioactivity remained associated with the cells following trypsinization of cells (Fig. 1, bottom panel).

Effects of Gangliosides on Superoxide Generation—GD3 (10 \(\mu\)M) stimulated the level of O\(_2^\cdot\) generation about 5-fold as compared with the control (Fig. 2). Other gangliosides like, GM1, GM2, and globotriosylceramide did not alter the O\(_2^\cdot\) level in these cells. LacCer also stimulated O\(_2^\cdot\) generation about 5-fold higher than the control and served as a positive control.

Effects of Time and Concentration of GD3 on Superoxide Production and GSH Levels—GD3 stimulated the generation of O\(_2^\cdot\) in H-ASMC in a concentration- and time-dependent manner (Fig. 3, A and B). The maximum stimulation of O\(_2^\cdot\) (>5-fold) was observed using (10 \(\mu\)M) GD3 (Fig. 2A), but at a relatively higher concentration of GD3 (50–200 \(\mu\)M) the level of O\(_2^\cdot\) was below the base-line value (Fig. 3A). Time kinetics studies reveal a lag time of about 2.5 min in O\(_2^\cdot\) production in cells stimulated by GD3. Subsequently, O\(_2^\cdot\) production increased linearly with time and attained a peak level at about 10 min of incubation with GD3 (10 \(\mu\)M). Thereafter, O\(_2^\cdot\) production moderately decreased...
in these cells. The preincubation of cells with antioxidant PDTC completely abrogated GD3 induced $O_2^-$ generation (Fig. 3C). However, superoxide dismutase partially inhibited GD3-induced $O_2^-$ levels (data not shown). These findings suggest that GD3-induced $O_2^-$ levels were both endogenous and exogenous.

Interestingly, as the concentration of GD3 in the incubation mixture increased, it was accompanied by a marked decrease in the level of GSH in these cells (Fig. 3D). A 50% decrease in the GSH level occurred in cells incubated with 25 $\mu$M GD3, and this level was sustained up to 200 $\mu$M (Fig. 3D).

Effect of GD3 on NAPDH/NADH Oxidase Activity—Cell membranes were prepared at varying time intervals after being incubated in A-SMC with/without GD3 (10 $\mu$M) and NAPDH/NADH oxidase activity was measured. Following a lag time of about 2.5 min, NAPDH/NADH oxidase activity increased linearly up to 10 min in cells incubated with GD3 (Fig. 4, A and B). The maximum increase in NAPDH oxidase activity (4-fold compared with control) was observed after 10–15 min incubation of cells with GD3 (Fig. 4A) and reached a plateau. Thereafter, there was no NAPDH oxidase activity observed in the cytosolic fraction in cells incubated with and without GD3 (data not shown). GD3 maximally increased (1.8-fold) the activity of NADPH oxidase in H-ASMC as compared with the control (Fig. 4B) following incubation for 10 min. These time data were obtained using 10 $\mu$M GD3. However, at a relatively higher concentration (100 $\mu$M), GD3 decreased the activity of NADPH oxidase to approximately one-half the control value (data not shown). And this may in part explain a decrease in $O_2^-$ production in A-SMC incubated with 100 $\mu$M GD3 (Fig. 3A).

Effect of GD3 on MAP Kinase Activity and Cell Proliferation—Fig. 5A represents the phosphorylation of the myelin basic protein peptide (APRTPGGRR) by immunoprecipitated cell lysate following stimulation with various concentrations of GD3. A concentration-dependent increase in MAPK activity was observed up to a concentration of about 10 $\mu$M GD3. However, we observed a progressive decline in the level of phosphorylated MBP in cells incubated with 50–200 $\mu$M of GD3 (Fig. 5B). In fact, at a concentration of 100 $\mu$M GD3, MBP phosphorylation fell below the control value. As shown in Fig. 5B, GD3 (10 $\mu$M) exerted a time-dependent stimulation in the activity of MAPK. Interestingly, preincubation of cells with cell-permeable antioxidant N-acetylcysteine and PDTC abrogated GD3 (10 $\mu$M) induced MAPK activity (Fig. 5C). GD3 (10 $\mu$M) exerted about a 5-fold increase in the incorporation of $[^3H]$thymidine in A-SMC, an index of cell proliferation (Fig. 5D). Preincubated cells with PDTC and NAC completely abrogated GD3-induced increase in $[^3H]$thymidine incorporation.

Effect of GD3 Concentration on Nitric Oxide in A-SMC—Following the incubation of A-SMC with 10 $\mu$M GD3 for 12 h, there was no production of NO (Fig. 6A). However, at higher concentrations (50–200 $\mu$M) GD3 markedly stimulated NO production. A maximum of 5-fold stimulation in NO production was observed with 100–200 $\mu$M GD3 (relative to control); this was within the range of stimulation of NO by LPS (10 $\mu$g/ml) and interferon-γ (200 units/ml) for 24 h. GD3-induced NO production was completely inhibited by preincubation of cells with 200 $\mu$M Nω-monomethyl-L-arginine (NMLA) (Fig. 6B). The incubation of A-SMC with GD3 (0–10 $\mu$M) exerted no effects on apoptosis/DNA ladder formation (Fig. 6C). However, at higher concentration (100–200 $\mu$M) GD3 markedly stimulated the intranucleosomal DNA degradation after 24 h, which was abrogated by preincubation of cells with NMLA (200 $\mu$M) (Fig. 6C).

Effect of GD3 on the Release of Cytochrome c—At a concentration of 10 $\mu$M, GD3 did not induce the release of cytochrome c from the mitochondria to cytosol. However, as we increased the concentration (50–200 $\mu$M) of GD3, a progressive increase in the level of cytochrome c release in cytosol was observed (Fig. 7). As a control we used C2-ceramide (1 $\mu$M), which also stimulated the release of mitochondrial cytochrome c in these cells.

**DISCUSSION**

Although glycosphingolipids have been shown to be present in mammalian cells their functional role in cell proliferation, adhesion and programmed cell death (apoptosis) are only beginning to emerge (1, 2, 26, 27). GD3 is a disialoganglioside that is enriched in human aortic smooth muscle cells. However, in pathological conditions such as in human melanomas and in atherosclerotic plaques, the level of GD3 is markedly elevated.
In the present study we explain signaling mechanisms by which GD3 can induce aortic smooth muscle cell proliferation as well as apoptosis. Previous studies employing subcellular fractionation, cell surface labeling, and electron microscope techniques have shown that gangliosides are predominately associated with the plasma membrane (27). The employment of freeze-fracture techniques revealed that globotriosylceramide is localized as microaggregates on the surface of erythrocytes (28). Employing [3H]GD3 in this study we have shown that during early time periods (10–30 min) of incubation, this ganglioside was associated mainly with a trypsin-sensitive cell surface component. Our studies confirm a previous report in human skin fibroblasts in which [3H]GM1 radioactivity was also found to associate with human skin fibroblast in a trypsin-sensitive state. However, upon long term (24 h or more) incubation [3H]GM1 radioactivity was also found to associate with human skin fibroblast in a trypsin-sensitive state. This speculation is based on a previous observation that a novel carbohydrate-glycosphingolipid interaction occurs between a β-(1–3) glucan immunomodulator, PGG-glucan, and lactosylceramide in human leukocytes (30).

The relevance of the uptake of [3H]GD3 kinetics with free radicals and cell signaling became evident when we observed that within 5 to 10 min of incubation with GD3 a 2-and 3-fold increase in the generation of superoxide occurred (Fig. 3). These findings suggest that the association of exogenously supplied GD3 to A-SMC cell surface was critical for the production of superoxide. This was accompanied by the activation/phosphorylation of MAPK and other downstream events that collectively contributed to A-SMC proliferation. This phenotypic change could be abrogated by preincubation of cells with NADPH oxidase inhibitor; diphenylene iododium, scavenger of free radicals; N-acetylcysteine; and a membrane permeable antioxidant such as PDTC (15). It may also be possible that GD3 molecules inserted into the plasma membrane (and which may well be in the range of about 1 mol%) are responsible for the formation of ROS, in particular in view of its likely distribution in so called rafts, where its concentration would be even...
higher. On the other hand, a cross-linking of surface proteins by surface-adhering GD3 micelles may evoke these effects by mimicking transmembrane signaling.

Why the exogenous supply of GD3 is required to bring about the generation of superoxide and cell proliferation when A-SMC have significant levels of endogenous GD3 is not clear to us at the present time. However, we have conducted preliminary studies employing wild type Chinese hamster ovary cells and mutant cells (SPB-1) that are devoid of LacCer and GD3 to address this issue. We observed that the basal level of superoxide generated (without exogenous supply of LacCer/GD3) in wild type and mutant cells was very low. However, upon the addition of exogenous LacCer/GD3 (10 μM) the mutant cells and wild type cells produced a 2–5-fold higher level of O₂⁻ as compared with control. Thus our preliminary studies revealed that exogenously supplied LacCer/GD3 stimulates O₂⁻ generation and may contribute to phenotypic changes in mammalian cells. A detailed description of our studies on wild type and mutant Chinese hamster ovary cells will be published elsewhere.²

Lipid second messenger such as LacCer and GD3 have been shown to be produced in cells upon the activation of glycosphin-golipid glycosyltransferases by cytokines. For example, we showed that incubation of human umbilical vein endothelial cells with TNF-α activated LacCer synthase and generated LacCer. In turn, LacCer activated NADPH oxidase to generate superoxide that was critical in the expression of intercellular cell adhesion molecule (ICAM-1) and the adhesion of neutrophils to human umbilical vein endothelial cells (31). This may also contribute to the process of apoptosis. Our studies indicate that at higher concentration, GD3 exerts a concentration-dependent decrease in the level of GSH. This is most likely explained by the observation that ceramide may be converted to GD3, which in turn may have contributed to apoptosis (see below).²

Previously, the release of cytochrome c from mitochondria to cytosol has been implicated in the apoptotic process. Interestingly, a cell-permeable ceramide (C₂₃-ceramide) also stimulated the release of cytochrome c in our studies but did not generate ROS or NO. This is most likely explained by the observation that ceramide may be converted to GD3, which in turn produces NO to induce apoptosis (11). Collectively, these lines of evidence provide strong support for the hypothesis that GD3 recruits NO to induce apoptosis in cultured H-ASMC.

Reduced GSH is a tripeptide antioxidant, which is present in all mammalian cells at a concentration between 1 and 10 mM (34). The predominant role of GSH is to provide a reduced environment inside the cell and to protect cells from redox stress. Our studies indicate that at higher concentration, GD3 exerts a concentration-dependent decrease in the level of GSH. This may also contribute to the process of apoptosis.

Because apoptosis can be induced in a number of cell systems by hydrogen peroxide, we measured the effects of GD3 on hydrogen peroxide levels. We found that although at low concentrations (10 μM), GD3 stimulated hydrogen peroxide levels, at high concentrations (100–200 μM) it reduced the cellular level of hydrogen peroxides (data not shown). Thus, these studies suggested that GD3 might not employ hydrogen peroxide or superoxide to induce death. On the other hand, previous studies have shown that exposure of cells to a variety of agonists such as pyrogallol, a generator of ROS, increases the DNA binding activity of NFkB that is followed by an increase in an inducible nitric-oxide synthase mRNA (35). Elevated levels of nitric oxide, in turn, stimulated apoptosis. This tenet was substantiated further by determining the effects of GD3 on the levels of nitric oxide by directly measuring the levels of nitrate

² A. Bhunia, G. Sato, and S. Chatterjee, unpublished observations.
as well as inducible nitric-oxide synthase levels by Western immunoblot assays. Although GD3 did not induce inducible nitric-oxide synthase in A-SMC and nitrite production at low

FIG. 5. Effect of GD3 on MAP kinase activity and cell proliferation. Following stimulation of cells with GD3, cell lysate was prepared and immunoprecipitated as described under “Materials and Methods.” Activity assay of MAPK was done by MBP (sequence APRTPGGR) phosphorylation. A, MBP phosphorylation by various concentrations of GD3. B, MAPK activity at different time intervals measured by direct counting vehicle (○) (10 μM) and GD3 (10 μM) stimulated (●) cells. C, assay of MAPK activity following incubation with NAC (15 mM) for 30 min, PDTC (100 μM) for 1 h, followed by incubation with 10 μM GD3. D, cells were incubated with 10 μM GD3 for 18 h. Next, [3H]thymidine incorporation was measured as described under “Materials and Methods.” In some experiments cells were preincubated with 100 μM PDTC for 1 h or 15 mM NAC for 30 min before incubation with 10 μM GD3 for 18 h. Each point is the mean ± S.D. of three separate experiments.

FIG. 6. Effect of GD3 on nitric oxide production and apoptosis. Following incubation with different concentrations of GD3 as indicated for 24 h, nitrite production was measured by greiss reagent (A). □, vehicle; ●, GD3. B, NMLA inhibited GD3-induced NO production. C, DNA ladder formation by different concentrations of GD3, as indicated.

FIG. 7. Effect of GD3 on cytosolic cytochrome c concentration. Cytosolic cytochrome c concentration was measured as described under “Materials and Methods” using different concentrations of GD3.
concentrations, at high concentrations, GD3 stimulated nitric oxide production. Previous studies have indicated that nitric oxide serves as a scavenger of \( O_2^- \) and forms toxic peroxynitrite (ONOO\(^{-} \)) radicals with endogeneous \( O_2^- \), which induces apoptosis. Accordingly, we can speculate that at high concentrations, GD3 generates NO that reacts with the endogenous \( O_2^- \). That markedly decreases the cellular level of \( O_2^- \) to produce toxic ONOO\(^{-} \). In turn, ONOO\(^{-} \) induces apoptosis. This mechanism may also explain why the level of \( O_2^- \) decreases as a consequence peroxynitrite. Clearly, additional studies need to be pursued to assess the physiological relevance of GD3 concentration (Fig. 3A) in addition to a decrease in NAPDH oxidase activity.

In summary, nonstimulated SMCs produce very low levels of \( O_2^- \) for the maintenance of normal growth and function but do not produce any NO. However, during the progressive phase in atherosclerosis, GD3 may stimulate cell proliferation via induction of \( O_2^- \) generation. In advanced atherosclerosis, when large amounts of GD3 have been found to be associated with plaque intima, it may lead to the generation of nitric oxide and subsequently peroxynitrite. Clearly, additional studies in vivo need to be pursued to assess the physiological relevance of GD3-mediated phenotypic changes in vitro in human aortic smooth muscle cells.

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