Disialoganglioside (GD3) Synthase Gene Expression Suppresses Vascular Smooth Muscle Cell Responses via the Inhibition of ERK1/2 Phosphorylation, Cell Cycle Progression, and Matrix Metalloproteinase-9 Expression*

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Sung-Kwon Moon§§, Hong-Man Kim¶, Young-Choon Lee‡, and Cheorl-Ho Kim‡‡

From the National Research Laboratory for Glycobiology, Ministry of Science and Technology, and Department of Biochemistry and Molecular Biology, Dongguk University College of Oriental Medicine, Kyungju City, Kyungbuk 780-714, Korea and the Faculty of Biotechnology, Dong-A University, Busan 604-714, Korea

Sialic acid-containing glycosphingolipids (gangliosides) have been implicated in the regulation of various biological phenomena such as atherosclerosis. Recent report suggests that exogenously supplied disialoganglioside (GD3) serves a dual role in vascular smooth muscle cells (VSMC) proliferation and apoptosis. However, the role of the GD3 synthase gene in VSMC responses has not yet been elucidated. To determine whether a ganglioside is able to modulate VSMC growth, the effect of overexpression of the GD3 synthase gene on DNA synthesis was examined. The results show that the overexpression of this gene has a potent inhibitory effect on DNA synthesis and ERK phosphorylation in cultured VSMC in the presence of PDGF. The suppression of the GD3 synthase gene was correlated with the down-regulation of cyclinE/CDK2, the up-regulation of the CDK inhibitor p21 and blocking of the p27 inhibition, whereas up-regulation of p53 as the result of GD3 synthase gene expression was not observed. Consistently, blockade of GD3 function with anti-GD3 antibody reversed VSMC proliferation and cell cycle proteins. The expression of the GD3 synthase gene also led to the inhibition of TNF-α-induced matrix metalloproteinase-9 (MMP-9) expression in VSMC as determined by zymography and immunoblot. Furthermore, GD3 synthase gene expression strongly decreased MMP-9 promoter activity in response to TNF-α. This inhibition was characterized by the down-regulation of MMP-9, which was transcriptionally regulated at NF-κB and activation protein-1 (AP-1) sites in the MMP-9 promoter. Finally, the overexpression of MMP-9 in GD3 synthase transfectant cells rescued VSMC proliferation. However, MMP-2 overexpression was not affected by cell proliferation. These findings suggest that the GD3 synthase gene represents a physiological modulator of VSMC responses that may contribute to plaque instability in atherosclerosis.

Vascular smooth muscle cell (VSMC) proliferation plays an important role in the development and progression of many cardiovascular diseases, including atherosclerosis (1). It is widely believed that platelet-derived growth factor (PDGF) stimulates the proliferation of vascular smooth muscle cells (2, 3). PDGF induces the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), a key transducer of extracellular signals that promotes cell growth and movement, which are critical for the initiation and progression of vascular lesions (4, 5).

In arterial media, VSMC are normally quiescent, proliferate at low indices (<0.05%), and remain in the G0/G1 phase of the cell cycle (6). After injury to the vessel, SMC migrates into the intimal layer of the arterial wall, where they leave their quiescent state and reenter the cell cycle (1). In many cells, transit through G1 of the cell cycle and entry into the S phase requires the binding and activation of cyclin-CDK complexes, a process in which cyclin D1-CDK4 and cyclin E-CDK2 play major roles (7, 8). The kinase activities of the cyclin-CDK complexes are negatively regulated by CDK inhibitors, such as p21 and p27 (9, 10). It is clear that p21 also plays an essential positive role in the assembly of certain cyclin-CDK complexes (11–15).

The expression of matrix metalloproteinase-9 (MMP-9) has been implicated in the progression of atherosclerotic lesions (4, 16). Recent reports, based on an in vivo study concluded that MMP-9 is critical for the development of arterial lesions via its regulation of both VSMC migration and proliferation (17, 18). On the basis of in-depth reports from several different laboratories, it has generally been concluded that the basal levels of MMP-9 are usually low, and that its expression can be induced by treatment of vascular smooth muscle cells with tumor necrosis factor-α (TNF-α) (19–21). Recent results have demonstrated that ERK1/2 mediates TNF-α-induced matrix metalloproteinase-9 expression in vascular smooth muscle cells via the regulation of NF-κB and AP-1 (20, 22).

Gangliosides, sialic acid-containing glycosphingolipids (GSL), play a role in the control of cell growth regulation through modulation of transmembrane signaling (23–25). In analyses of ganglioside expression in various human tumors, characteristic patterns have been demonstrated depending on

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§ Present address: Division of Food and Biotechnology, Chungju National University, Chungbuk 380-702, Korea.

¶ To whom correspondence should be addressed: NRL-Glycobiology and Dept. of Biochemistry and Molecular Biology, Dongguk University, Sukjang-Dong 707, Kyungju City, Kyungbuk 780-714, Korea. Tel.: 82-54-770-2663; Fax: 82-54-770-2281; E-mail: chkimbio@dongguk.ac.kr.

‡‡ The abbreviations used are: VSMC, vascular smooth muscle cell; PDGF, platelet-derived growth factor; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; FACS, fluorescent-activated cell sorter; DTT, dithiothreitol; GD3, disialoganglioside; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase.
individual tumor types, such as melanomas (26, 27), sarcomas (28), astrocytomas (29), and epithelial cancers (30). Among the tumor-associated glycolipids, disialyl ganglioside GD3 is thought to be a human melanoma-specific antigen (31) and has been used as a target molecule in antibody immunotherapy (32). It has been reported that the GD3 synthase gene expression results in the proliferation of PC12 cells through the Ras/MEK/ERK pathway (33). On the other hand, the overexpression of GD3 synthase led to apoptosis in human leukemic cells (34). In the past few years, increased levels of GD3 have been found to be associated with proliferative disease, such as atherosclerosis (35, 36). A recent study suggested that exogenously supplied GD3 has a dual role in modulating VSMC proliferation and apoptosis (37). However, the precise molecular mechanisms involved in GD3 synthase gene-mediated VSMC phenotypic changes have not been elucidated.

Recently, a number of siataltransferase genes were isolated in our group, and their molecular mechanism and expression patterns were investigated (46–49). The availability of these transferase genes enables further analysis of the implications of phenotypes of VSMC in atherosclerosis. The purpose of the present study was to determine the role of the GD3 synthase gene expressed on VSMC and the regulatory mechanisms involved in its expression.

EXPERIMENTAL PROCEDURES

Materials—PDGF-BB and TNF-α was obtained from R&D systems (Minneapolis, MN). Polyclonal antibody to cyclin D1, cyclin E, CDK2, CDK4, p21, p53, and p27 were obtained from New England Biolabs (Beverly, MA). Polyclonal MMP-9 antibody was obtained from Chemicon (Temecula, CA). Anti-GD3 monoclonal antibody was from Seikagaku Co. (Tokyo, Japan). Anti-GD3 monoclonal antibody was from Seikagaku Co. (Tokyo, Japan). Anti-GD3 monoclonal antibody was from Seikagaku Co. (Tokyo, Japan).

Cell Cultures—Mouse aortic smooth muscle cells were obtained from young (4-month-old) male rats by enzymatic digestion, as previously described in detail (38). Cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cultures were maintained in an atmosphere of humidified 95% air, 5% CO2 at 37 °C. For most experiments, cells at 80–90% confluence were made quiescent by incubation for 72 h in DMEM containing 0.1% fetal bovine serum.

Plasmid Construction and Cell Transfection—To obtain the full-length cDNA of GD3 synthase, first-strand cDNA synthesis was performed with the SuperScript™ preamplification kit (Invitrogen) according to the manufacturer’s instructions using 5 μg of total RNA from human adult brains (Clontech, Palo Alto, CA). The full-length cDNA was obtained by PCR with first-strand cDNA as a template using a 5′-primer containing a HindIII site, 5′-CTAAGCTTATGGCTGTAC-TG GGGTGGAAGTTCCCGCGG-3′ and a 3′-primer containing a Xhol site, 5′-ATCTCGAGTCCTAGGAAGTGGGCTGGAGTGAGGTATC-TC-3′. PCR was performed as follows: 94 °C for 60 s, 35 cycle of 94 °C for 60 s, 55 °C for 40 s, 72 °C for 90 s, and 72 °C for 10 min. The PCR product (1.1 kb) was cloned into pGEMBlue/R T-vector (Novagen, Inc., Madison, Madison) and determined by DNA sequencing. The inserted fragment (1.1 kb) was cut by digestion with HindIII and Xhol, and then inserted into the corresponding sites of pcDNA3 (Clontech, Palo Alto, CA), which was designated pcDNA3-GD3.

VSMC were transfected with pcDNA3-GD3 or pcDNA3 (no insert) in 100-mm dishes using the Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer’s protocol. After 24 h, cells were split at a 1:5 dilution and exposed for 2–3 weeks in G418 (Roche Applied Science)—containing medium (800 μg/ml), and the resulting colonies were selected based on their resistance to G418. The expression of GD3 synthase was confirmed by reverse transcriptase-PCR and immunoblot analysis using anti-GD3 monoclonal antibody against GD3 synthase gene expression with a Taqman sequence detection assay (PE Biosystems). In this assay, we used primers (5′-GAGCATGGTGGATGAGCGGGA-3′ and 5′-CTCAGGATGGCTGTCCTGGT-3′) to detect the specific GD3 synthase gene PCR product as it accumulates during PCR at the 60 °C annealing temperature. Similarly, the same RNA samples were PCR-amplified for β-actin, and the GD3 synthase gene expression was normalized to the β-actin.

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\text{P}^{32}\text{H} \text{Thymidine Incorporation} - \text{For } \text{P}^{32}\text{H} \text{thymidine uptake experiments, SMC were grown to near confluence in 24-well tissue culture plates and then made quiescent and treated with 10% FBS, as indicated. Cells were incubated for an additional 24 h and labeled with } \text{methyl-}{^3} \text{H} \text{thymidine (PerkinElmer Life Sciences) at } 1 \mu \text{Ci/ml during the last 24 h of this time period. After labeling, the cells were washed with phosphate-buffered saline and fixed in cold 10% trichloroacetic acid and then washed with 95% ethanol. Incorporated } \text{P}^{32}\text{H} \text{thymidine was extracted in 0.2 ml NaOH and measured by liquid scintillation counter as previously described (20, 38).}
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Cell Cycle Analysis (FACS)—Cells were harvested and fixed in 70% ethanol and stored at −20 °C. Cells were then washed twice with ice-cold phosphate-buffered saline, incubated with RNase and DNA intercalating dye propidium iodide, and cell cycle phase analysis was performed by flow cytometry using a BD Biosciences FACStar flow cytometer and Becton Dickinson cell fit software.

Immunoblotting—Growth-arrested VSMCs were treated with 10% FBS for the specified time periods at 37 °C. Cell lysates were prepared, and immunoblotting was performed as described previously (20, 38).

Immunoprecipitation and Immune Complex Kinase Assays—Cell lysates were prepared with ice-cold lysis buffer (containing, in mM, HEPES (pH 7.5) 50, NaCl 150, EDTA 1, EGTA 2.5, DTT 1, β-glycerophosphate 10, NaF 1, Na3VO4 0.1, phenylmethylsulfonyl fluoride 0.1, and 10% glycerol, 0.1% Tween-20, 10 μg/ml of leupeptin, and 2 μg/ml of aprotinin) and sonicated at 4 °C (Micro ultrasonic cell disrupter (from KONITES), 30% power, two times for 10 s each time). Lysates were clarified by centrifugation at 10,000 × g for 5 min, and the supernatants were precipitated by treatment with protein A-Sepharose beads pre-coated with saturating amounts of the indicated antibodies at 4 °C for 2 h. When monoclonal antibodies were used, protein A-Sepharose was pretreated with rabbit anti-mouse immunoglobulin G (Jackson ImmunobioResearch Laboratories). The immunoprecipitated proteins on the beads were washed four times with 1 ml of lysis buffer containing a kinase buffer (containing, in μg/liter, HEPES 50, MgCl2 10, DTT 1, β-glycerophosphate 10, NaF 1, and sodium orthovanadate 0.1). The final pellet was resuspended in 25 μl of kinase buffer containing either 1 μl of glutathione S-transferase (GST)-pRb C-terminal (pRB amino acids 769–921) fusion protein (Santa Cruz Biotechnology) or 5 μg of histone H1 (Invitrogen), 20 μg/liter ATP, and 5 μCi of [γ-32P]ATP (4500 μCi/mmol; ICN) and incubated for 20 min at 30 °C with occasional mixing. The reaction was terminated by the addition of 25 μl of 2× concentrated Laemmli sample buffer and separated on 10 or 12.5% SDS-polyacrylamide gels. The migration of histone H1, or GST-pRb was determined by Coomassie Blue staining. Phosphorylated pRB and histone H1 were visualized.

Zymography—Conditioned medium and cell lysates were electrophoresed in a polyacrylamide gel containing 1 mg/ml gelatin. The gel was then washed at room temperature for 2 h with 2.5% Triton X-100 and subsequently in a buffer containing 10 μM CaCl2, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5 at 37 °C overnight. The gel was stained and photographed with Coomassie Blue (Sigma) for gelatinase bands (staining for gelatinase A and B). Proteolysis was detected as a white zone in a dark blue field.

MMP-9 Promoter Activity—A 0.7 kb segment at the 5′-flanking region of the human MMP-9 gene was amplified by PCR using specific primers from the human MMP-9 gene (GenBank™ accession no. M10887): 5′-ACATTTGCGAGGTCACTGAAGforward/SacIand 5′-AAGGGGCTTCCAGATCTGGTCCCTGC reverse vector containing a polyadenylation signal upstream from the luciferase gene was used to construct expression vectors by subcloning PCR-amplified DNA of the MMP-9 promoter into the SacI/HindIII site of the pGL2-Basic vector. The PCR products were confirmed by their size as...
Role of GD3 Synthase Gene in Vascular Smooth Muscle Cells

Overexpression of GD3 Synthase Gene Inhibits VSMC Proliferation—Induction of the GD3 synthase gene expression in parental cells (Un), vector control cells (EV), and cells stably expressing GD3 synthase gene (GD3). A quantitative reverse transcriptase-PCR amplification of MMP-9 mRNA level of indicated cell lines. Steady state mRNA levels for the GD3 synthase gene were normalized to β-actin B. Cell lysates from the indicated cell lines were examined by immunoblot analysis with anti-GD3 antibody. The results were normalized to GAPDH expression. C, indicated cell lines were incubated in serum-free medium for 24 h, treated with or without PDGF (10 ng/ml) for 24 h, and labeled with [3H]-thymidine during the last 24 h of this time period. Thymidine incorporation was assessed by scintillation counting of precipitated DNA. Similar results were observed in three independent experiments.

RESULTS

GD3 Synthase Gene Overexpression Inhibits DNA Synthesis in VSMC—To examine the role of GD3 in PDGF-mediated VSMC, VSMC were transfected with a cDNA (GD3) encoding GD3 synthase gene or an identical empty vector lacking a cDNA insert as a control (EV). Stable cell clones were selected by adding G418, at a concentration of 400 μg/ml to the culture medium. As an additional control, parental cells (Un) were evaluated in an identical manner. Expression of GD3 synthase gene was verified by reverse transcriptase-PCR and immunoblot (Fig. 1A and B). We evaluated the effects of GD3 synthase gene overexpression on VSMC proliferation in response to PDGF. PDGF-mediated DNA synthesis was measured by [3H]-thymidine incorporation. PDGF induced significant increases in DNA synthesis in both parental and EV-transfected VSMC (Fig. 1C). However, GD3-synthase transfectants almost blocked the PDGF-induced increase in DNA synthesis.

GD3 Synthase Gene Induces G1 Cell Cycle Arrest—Cell DNA content was measured by FACS to determine the cell cycle distribution under various culture conditions. To synchronize the cell cycle, cells were initially deprived of FBS for 24 h and subsequently incubated with PDGF for 24 h. GD3 synthase transfectant cells showed no change in cell cycle distribution compared with the EV transfectant cells in the absence of 10% FBS (Fig. 2B, panels a and e). When stimulated by PDGF, the GD3 synthase transfectants exhibited a significantly increased the cells arrested in G1/G0 stages, whereas EV transfectants had reached the S and G2/M stages respectively (Fig. 2B, panels b and d). To clarify the induction of apoptosis during the growth suppression of GD3 synthase transfectant cells, a cell death ELISA quantitative assay was performed. No differences were found in the rates of cytoplasmic DNA-histone complex formation in the GD3 synthase and EV transfectant cells (Fig. 2A), indicating that thymidine uptake represent differences in proliferation rather than cell death.

GD3 Synthase Transfectant Cells Block ERK1/2 Phosphorylation and Modulate G1 Cell Cycle-associated Proteins—To analyze whether the GD3 synthase gene is able to inhibit the phosphorylation of ERK1/2, we next examined the MAP kinase signaling pathway ERK1/2 after PDGF stimulation in GD3 synthase transfectant, EV transfectant, and parental cells. As shown in Fig. 3A, the overexpression of the GD3 synthase gene inhibited PDGF-mediated ERK1/2 phosphorylation, suggesting that GD3 overexpression could have an effect on VSMC proliferation by inhibiting ERK1/2 activation. To determine if GD3 synthase gene overexpression in VSMC proliferation is associated with the decreased activation of cell cycle machinery, we examined the expression of cell cycle regulatory molecules using immunoblot and kinase assay. The expression of G1-associated factors, cyclin E and CDK2, were down-regulated in GD3 transfectant cells after PDGF treatment as compared with the EV transfectant or parental cells (Fig. 3B). However, overexpression of the GD3 synthase gene in VSMC had no effect on the levels of other G1 cell cycle proteins, cyclin D1 and CDK4. The kinase activities associated with the CDKs are the driving forces for the progression of the cell cycle through the transition checkpoints. Therefore, we assessed the kinase activities associated with CDK2 and CDK4 in GD3 synthase gene transfectant, EV transfectant, and parental cells. CDK complexes were immunoprecipitated using specific anti-CDKs antibodies, and the levels of CDK-associated kinase activity were measured against the Rb protein or histone H1 as the substrate. CDK2 kinase activity was markedly suppressed after treatment with PDGF in the VSMC expressing the GD3 synthase gene compared with the EV transfectant or parental cells (Fig. 4A). Overexpression of GD3 synthase gene in VSMC had no effect on CDK4 kinase activities.

Overexpression of GD3 Synthase Gene Modulates p21 and p27 Levels—We next assessed the effect of the GD3 synthase gene on the induction of p21 and p27, which are known to regulate the entry of cells at the G1 to S phase transition checkpoint. Immunoblot analysis revealed that GD3 synthase gene transfectants, after treatment with PDGF, resulted in a significant induction of p21, compared with the parental cells and EV-transflectant cells.
In particular, even the basal levels, p21 levels were increased in GD3 transfectant cells. Importantly, overexpression of the GD3 synthase gene blocked the inhibition of p27, as induced by PDGF (Fig. 4B). However, under similar experimental conditions, the level of expression of the p53 tumor suppressor protein was unchanged.

**Reverse of Decreased Cell Proliferation by anti-GD3 Antibodies**—Effects of anti-GD3 antibodies on cell proliferation were then examined by adding antibodies to the culture medium. The suppressed cell proliferation after GD3 synthase gene transfection was strongly reversed in the presence of anti-GD3 antibodies. These effects were dependent on the concentration of the added antibody, and became significant, even at 5 μg/ml on 24 h (Fig. 4B). In EV transfectants, addition of an anti-GD3 antibody did not affect cell proliferation and ERK1/2 phosphorylation (Fig. 4A and B). Consistently, blockade of GD3 with anti-GD3 antibodies reversed cell cycle-associated proteins including cyclin E, CDK2, p21, and p27 levels in GD3 synthase transfectants (Fig. 5, A and B). These results suggest that the GD3 plays important roles in VSMC proliferation.

**Overexpression of GD3 Synthase Gene Suppresses TNF-α-induced MMP-9 Expression**—In the past several years, a number of studies have demonstrated that MMP-9 is important for VSMC proliferation and migration into the intima (4, 16). Recent studies have reported that TNF-α stimulates MMP-9 induction in VSMC (19–21). Further studies have demonstrated that MMP-9 is necessary for the regulation of smooth muscle...
cell replication after arterial injury (17, 18). Therefore, our observation of GD3 synthase gene-mediated changes in proliferative capacity, the ERK1/2 pathway and the cell cycle machinery in VSMC led us to examine TNF-\( \alpha \)-induced MMP-9 expression in GD3 synthase gene transfectant cells. The gelatinases secreted by parental, EV transfectants, and GD3 transfectant cell cultures were identified by the zymographic detection of gelatinolytic activity after TNF-\( \alpha \)-treatment (Fig. 7). The conditioned media from GD3 transfectant cells showed much less MMP-9 gelatinolytic activity, compared with parental cells. Moreover, the introduction of an empty vector had no effect on TNF-\( \alpha \)-induced MMP-9 activity. Similar results were found in cell lysates and by immunoblot analysis (Fig. 7).

Overexpression of GD3 Synthase Gene Inhibits the MMP-9 Promoter by Decreasing the NF-\( \kappa \)B and AP-1 Binding Activities

In order to determine the mechanism for the decreased MMP-9 expression, we examined MMP-9 promoter activity before and after TNF-\( \alpha \)-stimulation in parental, EV transfectants, and GD3 synthase gene transfectant cells. In a previous study in our laboratory, we found that the minimal response elements, NF-\( \kappa \)B and AP-1, for TNF-\( \alpha \)-stimulation are located in the region \( \approx 710 \)-bp upstream of the transcription start site in VSMC (20, 22). In addition, a previous report showed that both sites are conserved in the human, mouse, and rat MMP-9 promoters (39). Therefore, we used a plasmid containing a luciferase reporter gene driven by a 710-bp segment from the 5'-promoter region of human MMP-9 gene to examine TNF-\( \alpha \)-mediated MMP-9 promoter activation in parental, EV transfectants, and GD3 synthase gene transfectant cells. VSMC were transiently transfected with plasmid pGL2-MMP-9WT (see “Experimental Procedures”), and subsequently treated with TNF-\( \alpha \) for 24 h. TNF-\( \alpha \) strongly increased reporter activity, which can be attributed to the MMP-9 promoter sequence in parental and EV transfectant cells (Fig. 8A). In addition, this TNF-\( \alpha \)-stimulated MMP-9 promoter activity was reduced by...
over 90% in GD3 synthase gene transfectant cells, suggesting that the repressive effect of the GD3 synthase gene is due, at least in part, to reduced transcription of the MMP-9 gene. As mentioned above, our previous studies showed that TNF-α and C stranded oligonucleotide probe with the consensus sequence for nuclear extracts were incubated with a radiolabeled double-stranded oligonucleotide probe, respectively. AP-1 binding activities were effectively suppressed in GD3 synthase transfectant cells (Fig. 8, B and C). In EMSA, nuclear extracts from the cells were analyzed by EMSA for the activated NF-κB and AP-1 using radiolabeled oligonucleotide probes, respectively.

**DISCUSSION**

A number of reports have appeared on the modulation of cell growth and differentiation by glycosphingolipids (23–25). In the past few years, several groups have reported an accumulation of gangliosides in atherosclerosis vessels (35, 36). Many of these studies were performed by adding exogenous glycolipids into the culture medium of cell lines under investigation. It has been shown that the addition of exogenous gangliosides to a culture medium inhibits cellular growth via the suppression of tyrosine phosphorylation of several growth factor receptors (23, 40). In particular, gangliosides, GM1, GM2, and GM3 inhibit the tyrosine phosphorylation of the PDGFβ receptor as well as of other early intracellular events and, therefore, lead to a reduction in the proliferative effects of PDGF-BB in VSMC (41). On the other hand, previous studies suggest that GM1 and GM2 induce VSMC proliferation via the ERK1/2 pathway (42). It has recently been shown that ganglioside GD3 has a dual function by stimulating proliferation and triggering the apoptosis of VSMC (37). Although a few studies have analyzed the effects of endogenously generated ganglioside GD3 on the functions of cell growth and differentiation in neuroblastoma (33) and PC12 cell lines (44), the molecular and cellular mechanisms underlying VSMC responses of the GD3 synthase gene transfectant cells remain unknown. In this study we demonstrate a novel mechanism of anti-atherogenic response of the GD3 synthase gene in VSMC.

We were not able to obtain sufficient GD3 synthase gene transfectant cells for a TLC analysis of total gangliosides, because GD3 synthase transfectants exhibited a very slow growth. However, the increased GD3 synthase gene and GD3 protein levels were verified by reverse transcriptase-PCR and immunoblot studies (Fig. 1, A and B). The transfection of the GD3 synthase gene into VSMC resulted in an observed decrease in extent of thymidine uptake (Fig. 1C). In addition, we demonstrated that VSMC expressing the GD3 synthase gene suppress PDGF-induced ERK1/2 phosphorylation. These results are consistent with previous findings indicating that gangliosides GM1, GM2, and GM3 inhibit the PDGF-induced signaling transduction pathway in VSMC (41). Our observations in this experiment are in general agreement with a previous report showing that high concentrations of GD3 inhibited ERK1/2 activity and cell proliferation (37), although other findings indicate that, at a low concentration, GD3 recruits superoxide to activate ERK1/2 and stimulates cell proliferation (37). Because GD3 is a major glycosphingolipid in normal aortic VSMC, and its level is markedly increased in human aortic plaque intima (35, 36), our findings may explain, in part, the GD3-associated transition to plaque instability in advanced atherosclerosis.

Several studies have identified gangliosides that are involved in regulation of cell growth and signaling pathway in various cell lines (23–25). However, the molecular mechanisms including cell cycle regulation and MMP-9 expression involved in ganglioside-mediated cell phenotypic changes have not been studied. We therefore investigated the involvement of the CKI-cyclin-CDK machinery during cell growth retardation by the GD3 synthase gene in VSMC. The G1 to S cell cycle progression is controlled by several CDK complexes, such as cyclinD1-CDK4 and cyclinE-CDK2, the activities of which are dependent on the balance of cyclins and CDK inhibitors (CKIs), such as p27 and p21. To investigate the issue of whether GD3 synthase gene-induced cell growth inhibition is caused by the down-regulation of cyclins and CDKs or the up-regulation of CKIs, we then analyzed the expression of these cell-cycle regulators in VSMC overexpressing the GD3 synthase gene. Our experiment indicated that the overexpression of the GD3 synthase gene on VSMC was found to result in a significant down-modulation of cyclin E-CDK2 in response to PDGF, although to a different extent. However, under similar experimental conditions, cyclin D1-CDK4 levels remained unchanged. The overexpression of...
the GD3 synthase gene led to an inhibition in the kinase activities associated with CDK2, but not CDK4. Our data also demonstrated a significant up-regulation of p21, during the G1-phase arrest of VSMC by the GD3 synthase gene. Interestingly, overexpression of GD3 synthase gene blocked the inhibition of p27 following PDGF. Many studies have shown that the regulation of G1 cell cycle arrest can be attributed to a number of cellular proteins, including p53 (45). However, the GD3 synthase gene had no effect on p53 protein levels in VSMC, as determined by immunoblot analysis, suggesting that the GD3 synthase gene induced accumulation of p21 and blocked inhibition of p27, as induced by PDGF, could be responsible for G1 phase arrest. To our knowledge, this is the first study showing the involvement of cyclins/CDKs/CKI machinery during cell growth inhibition induced by a ganglioside. We conclude that the ganglioside likely inhibits cell cycle progression through coordinate effects on positive regulators, cyclin E-CDK2, and negative regulators of CDKs, p21, and p27.

In our results, p21 protein levels were increased in response to PDGF (Fig. 4B). Recently, data have appeared to implicate the CDK inhibitor p21 in positive effects on cyclin-CDK activation in mammary epithelial cells, fibroblast cells, and VSMC (11–15). We propose that in addition to their roles as inhibitors, the p21 family of proteins, originally identified as inhibitors, may also play roles as adaptor proteins.

The effects of GD3 on VSMC proliferation and cell cycle-associated proteins were confirmed by adding anti-GD3 antibody. Consistent with our observations that overexpression of GD3 synthase gene inhibited cell proliferation and modulated cell cycle-associated proteins, blockade of GD3 function with the anti-GD3 antibody reversed VSMC proliferation and cell cycle-associated proteins. Gangliosides, including GD3, are expressed on the outer layer of plasma membranes (50). GD3 captured by the anti-GD3 antibody on the cell surface might cause clustering of GD3 molecules, resulting in the modulation of neighboring molecules and in the inactivation of subsequent intracellular molecules. These data provide evidence that ganglioside GD3 is the important mediator of anti-VSMC proliferation.

We next examined whether GD3 synthase gene overexpression inhibits TNF-α-stimulated MMP-9 activity in VSMC because the expression of MMP plays important roles in the pathogenesis of atherosclerosis and restenosis after vascular injury (4, 16). Of considerable interest in this study was the marked decrease in TNF-α-induced MMP-9 activity in VSMC expressing the GD3 synthase gene as determined by zymography and immunoblot analysis. Under the same experimental conditions, overexpression of the GD3 synthase gene had no effect on MMP-2 expression. Previous studies in our laboratory and others have demonstrated that TNF-α induced transcriptional MMP-9 promoter activity in VSMC through AP-1 and NF-κB (20, 22, 43). This result was confirmed by transfecting the pGL2-MMP-9WT plasmid, whose promoter region contains binding sites for NF-κB and AP-1 transcription factors. Consistent with the zymography and immunoblot analyses, our data showed that MMP-9 promoter activity is effectively suppressed by the GD3 synthase gene. Finally, we attempted to determine whether the decreased binding activities of AP-1 and NF-κB could account for the GD3 synthase gene-induced decrease in MMP-9 expression in VSMC. Using consensus AP-1 and NF-κB probes, a marked decrease in both AP-1 and NF-κB binding activities in response to TNF-α in GD3 synthase transfectant cells was observed (Fig. 8). Collectively, these findings provide support for a scenario in which AP-1 and NF-κB motifs in the MMP-9 promoter are associated with the transcriptional MMP-9 down-regulation in response to the GD3 synthase gene in VSMC. Finally, in order to investigate the effect of MMP-9 on GD3-induced cell growth inhibition, MMP-9 was transfected into GD3 synthase transfectants. Unexpectedly, GD3-induced cell growth inhibition, including DNA synthesis and ERK1/2 phosphorylation, were rescued by expressing MMP-9. Rescue of cell proliferation in GD3 synthase transfectants by overexpression of MMP-9, as demonstrated in the present study, should be the first example. The molecular mechanisms for the effect of MMP-9 in GD3 synthase transfectants are now under investigation in our laboratory and should provide information necessary for the application of GD3 in atherosclerosis.

In conclusion, this is the first study showing that GD3 synthase gene overexpression effectively suppresses cell proliferation, cell cycle progression, and MMP-9 expression in VSMC, suggesting that a ganglioside could be an effective candidate.

**Fig. 9. Effect of the MMP-9 and MMP-2 genes in GD3 synthase transfectants.** A and C, indicated cell lines were transfected with the MMP-9 and MMP-2 gene, respectively, then incubated in serum-free medium for 24 h, in the presence or absence of PDGF (10 ng/ml) for 24 h, and labeled with [methyl-3H]thymidine during the last 24 h of this time period. Thymidine incorporation was assessed by scintillation counting of precipitated DNA. Similar results were observed in three independent experiments. B, cell lysates from the indicated cell lines were examined by immunoblot analysis with anti-phospho-ERK1/2 antibody. The results were normalized to anti-ERK1/2 antibody expression.
for the prevention of vascular proliferative disorders. Thus, additional studies are required to establish the potency of GD3 synthase gene overexpression in vivo. Furthermore, GD3 synthase gene transfectant cells may be ideal models for elucidating the molecular and cellular events that lead to ganglioside-associated increases in advanced atherosclerosis.

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Sung-Kwon Moon, Hong-Man Kim, Young-Choon Lee and Cheorl-Ho Kim

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