

Suppression of vascular smooth muscle cell responses induced by TNF- α in GM3 synthase gene transfected cells

SUNG-SUK PARK¹, WUN-JAE KIM² and SUNG-KWON MOON¹

¹Department of Food and Biotechnology, Chungju National University, Chungju, Chungbuk 380-702; ²Department of Urology, Chungbuk National University College of Medicine, Cheongju, Chungbuk 361-763, South Korea

Received July 29, 2010; Accepted October 1, 2010

DOI: 10.3892/ijmm.2010.561

Abstract. The natural accumulation of ganglioside GM3 (N-glycolylneuraminic acid) on atherosclerotic lesions is a common theory. The present study is the first to examine the effects of the GM3 synthase gene on the responses of vascular smooth muscle cells (VSMC) to tumor necrosis factor- α (TNF- α). We found that overexpression of the GM3 synthase gene inhibited DNA synthesis and ERK1/2 activity induced by TNF- α in VSMC, whereas the basal levels of DNA synthesis and ERK1/2 activity remained unchanged. In addition, GM3 synthase gene transfectants significantly reduced the migration and invasion of VSMC following TNF- α treatment, compared with empty vector transfectants. Furthermore, TNF- α -induced matrix metalloproteinase-9 (MMP-9) expression and promoter activity were also decreased in GM3 synthase gene transfectants. GM3 synthase gene expression markedly suppressed the TNF- α -stimulated transcriptional activity of activator protein-1 (AP-1) and nuclear factor- κ B (NF- κ B), which are the controlling factors of MMP-9 expression. Consistent with these results, the addition of anti-GM3 antibody into the GM3 synthase gene transfectants blocked inhibition of DNA synthesis, ERK1/2 activity, migration and invasion. Finally, GM3 synthase gene transfectants treated with anti-GM3 antibody reversed the suppression of MMP-9 expression by reducing AP-1 and NF- κ B binding activity. These results suggest regulatory roles for the GM3 synthase gene in VSMC proliferation and migration during the formation of atherosclerotic lesions.

Introduction

The proliferation and migration of vascular smooth muscle cells (VSMC) from the blood vessel media into the intima

plays a fundamental role in the development and progression of vascular lesions including atherosclerosis and restenosis (1). Accumulation of inflammatory cells, which produce cytokines, contributes to the formation of vascular lesions through several pathophysiological processes (2). Tumor necrosis factor- α (TNF- α), a potent cytokine, is expressed in VSMC after balloon-induced injury of rat aortas (3-5). The presence of elevated levels of TNF- α has been demonstrated in the plaques of atherosclerotic arteries (6). TNF- α has been shown to influence VSMC proliferation via up-regulation of the extracellular signal-regulated kinase (ERK1/2) (7).

VSMC migration is a crucial event in the development of atherosclerotic lesions resulting from excessive vascular remodeling followed by matrix deposition (8). Migration of VSMC is facilitated by the proteolytic degradation of the extracellular matrix (ECM) surrounding VSMC after vascular injury, which contributes to neointimal formation (9). Matrix metalloproteinases (MMPs), such as the gelatinases MMP-2 (72 kDa) and MMP-9 (92 kDa), have been implicated in the proteolytic degradation of the ECM, during vascular lesion formation (9). Many studies have demonstrated that MMP-9 is a highly important factor in the progression of arterial lesions, where it may function to regulate both VSMC migration and proliferation (10-14). MMP-9 expression and activation is stimulated by TNF- α but not by the platelet-derived growth factor (PDGF) or thrombin in VSMC *in vitro* (10-12).

Gangliosides are sialic acid-containing glycosphingolipids (GSL) that are found in vertebrate plasma membranes (15). Numerous studies have demonstrated that gangliosides influence the regulation of various cellular functions, including cell proliferation, apoptosis, migration and invasion (16-18). Among the various gangliosides, the monosialosyl ganglioside GM3, the simplest ganglioside oligosaccharide, is thought to be strongly associated with malignant tumor cells (16,18-21). It has been reported that GM3 suppresses the proliferation and invasion of tumor and keratinocyte cells (16,18,21). Moreover, previous studies showed enhanced insulin sensitivity in animal models lacking the GM3 synthase gene (22). Several studies have shown the accumulation of GM3 in atherosclerotic lesions (23,24). In another study, exogenously-supplied GM3 inhibited VSMC proliferation caused by PDGF (25). Although many studies have analyzed the effects of the exogenously-supplied ganglioside GM3 in several cell lines, the cellular responses to GM3 synthase gene expression involved in the inhibition of cell proliferation, migration and invasion in

Correspondence to: Dr Sung-Kwon Moon, Department of Food and Biotechnology, Chungju National University, 123 Geomdan-ri Iryu-myeon, Chungju, Chungbuk 380-702, South Korea
E-mail: skmoon@cjnu.ac.kr

Key words: atherosclerosis, ganglioside, GM3 synthase gene, tumor necrosis factor- α , ERK1/2, matrix metalloproteinase-9, activator protein-1, nuclear factor- κ B, vascular smooth muscle cells

VSMC remain to be identified. In addition, there is no report in the literature regarding the modulation of TNF- α responses regulated by GM3.

In the present study, we provide new evidence that GM3 synthase gene expression inhibited cell proliferation, invasion and migration via regulating ERK1/2 activation and MMP-9 expression in TNF- α -treated VSMC.

Materials and methods

Materials. TNF- α was obtained from R&D Systems (Minneapolis, MN, USA). Polyclonal antibodies to ERK1/2 and phospho ERK1/2 were obtained from New England Biolabs (Beverly, MA, USA). Polyclonal MMP-9 antibody was obtained from Chemicon (Temecula, CA, USA). Anti-GM3 monoclonal antibody was obtained from Seikagaku Corp. (Tokyo, Japan).

Cell cultures. Mouse aortic smooth muscle cells were obtained from young (4-month old) male rat aortas by enzymatic digestion, as previously described in detail (26). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/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 24 h in DMEM without fetal bovine serum.

Plasmid construction and cell transfection. The cDNA of the mouse GM3 synthase was amplified from total RNA of HT22 cells using the following primers: forward, 5'-CCGGA TCCCAGCACAAAGATGA-3' and reverse, 5'-GTGGATCCG AGTTCGCTGTGGATG-3' based on the GenBank™ sequence information (AB018048). Purified polymerase chain reaction (PCR) products were subcloned into the pcDNA3.1 (Invitrogen) vector. The correct sequence and orientation of the constructs were confirmed by DNA sequencing.

VSMC were transfected with pcDNA3-GM3 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 were cultured for 2–3 weeks in G418-containing medium (800 μ g/ml) (Boehringer-Mannheim, Indianapolis, IN). The resulting colonies were selected based on G418 resistance. Expression of the GM3 synthase gene was confirmed by RT-PCR and immunoblot analysis using a monoclonal antibody against GM3.

RT-PCR. Cells from culture experiments were scraped and lysed in TRIzol (Gibco-BRL), and RNA was isolated by chloroform extraction and isopropanol precipitation. Total RNA (2 μ g) from each sample was treated with DNase I (Promega, Madison, WI), reverse-transcribed using Super Script Reverse Transcriptase (Promega), and quantitatively amplified for GM3 synthase gene expression using PCR with a Taqman Sequence Detection Assay (PE Biosystems). In this assay, forward and reverse primers (forward, 5'-CAG CACAAGATGAGAAGACCC-3', and reverse, 5'-GCGTAG TATTCACGTCCTGAC-3') were used to detect the unique

GM3 synthase gene PCR product as it accumulated during PCR at the annealing temperature of 60°C. The same RNA samples were PCR-amplified for β -actin using a similar method and GM3 synthase gene expression was normalized to the β -actin.

[³H]thymidine incorporation. For [³H]thymidine-uptake experiments, VSMC were grown to near confluence in 24-well tissue culture plates and then made quiescent and treated with thrombin, as indicated. Cells were incubated for an additional 24 h, during which time they were labeled with [methyl-³H]-thymidine (New England Nuclear Corp., Boston, MA, USA) at 1 μ Ci/ml. After labeling, the cells were washed with phosphate-buffered saline, fixed in cold 10% trichloroacetic acid, and then washed with 95% ethanol. Incorporated [³H]thymidine was extracted in 0.2 M NaOH and measured by a liquid scintillation counter as previously described (26,27).

Apoptosis assays. A sandwich ELISA method was used to assess apoptosis (Cell Death Detection ELISA, Roche Molecular Biochemicals) (27). The method is based on quantification of the enrichment of mono- and oligo-nucleosomes in the cytoplasm. Briefly, 1 \times 10⁴ cells in 100 μ l of DMEM were incubated as indicated lysed and centrifuged. Supernatants containing cytoplasmic histone-associated DNA fragments were transferred to a microplate coated with streptavidin, and then reacted with a mixture of biotinylated anti-histone antibody and anti-DNA antibody coupled to peroxidase. The peroxidase substrate was then added and the colored product was read photometrically at 405 nm using a reference wavelength of 490 nm. Specific enrichment of mono- and oligo-nucleosomes released into the cytoplasm was expressed as an enrichment factor relative to the control.

Immunoblotting. Growth-arrested VSMC were treated with TNF- α for specified time periods at 37°C. Cell lysates were prepared, and immunoblotting was performed as described previously (26,27).

Invasion assay. The VSMC were resuspended with TNF- α (100 ng/ml) alone or together with naringin in 100 μ l of medium and placed in the upper part of a Transwell plate. The cells were then incubated for 24 h. Cells had to pass through a polycarbonate membrane with an 8 μ M pore size and a thin layer of ECMatrix-like material. The ability of VSMC to invade the ECMatrix-like material was determined with a commercial cell invasion assay kit (Chemicon International), as described previously (28).

Wound migration assay. VSMC were plated on 6-well dishes and grown to 90% confluence in 2 ml of growth medium. Cells were wounded using a 2-mm-wide tip and then treated with TNF- α (100 ng/ml) alone or together with naringin. The cells were allowed to migrate, and photographs were taken through an inverted microscope (magnification \times 40).

Zymography. The conditioned medium was 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 at 37°C overnight in a buffer containing 10 mM

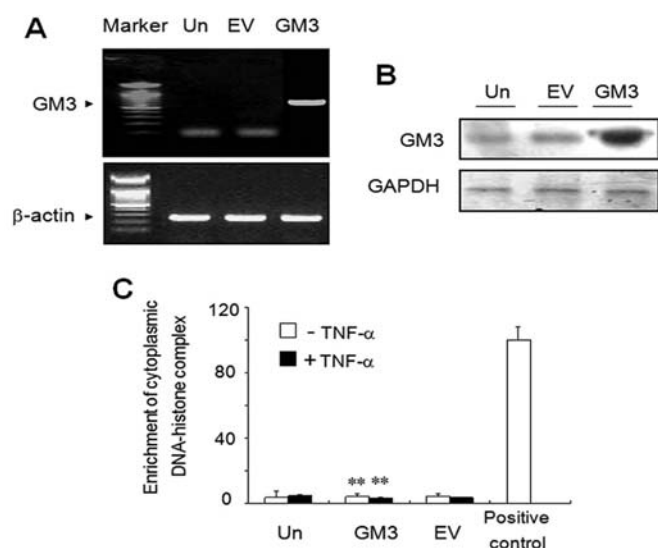


Figure 1. Expression levels of the GM3 synthase gene in transfected cells. GM3 synthase gene expression in parental cells (Un), empty vector transfected cells (EV) and cells endogenously expressing GM3 synthase gene (GM3). (A) Increased mRNA expression of GM3 synthase gene was detected by RT-PCR in GM3 synthase gene transfectants. Steady-state GM3 synthase gene mRNA was normalized to β -actin. (B) Immunoblot analysis was performed in cell lysates from established cell lines using anti-GM3 antibody. The results were normalized to GAPDH expression. (C) Apoptotic cells were quantified by histone-associated DNA fragmentation ELISA assay. Results are presented as means \pm SE from three triplicate experiments. ** $P < 0.01$ compared with control for each experiment.

CaCl₂, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5. The gel was stained with 0.2% Coomassie blue and photographed on a light box. Proteolysis was detected as a white zone in a dark blue field.

Creation of the MMP-9 promoter reporter construct. 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 (Accession No. D10051): 5'-ACATTTGCCC GAGCTCCTGAAG (forward/*Sac*I) and 5'-AGGGGCTGCC AGAAGCTTATGGT (reverse/*Hind*III). The pGL2-Basic vector containing a polyadenylation signal upstream from the luciferase gene was used to construct the expression vectors by subcloning PCR-amplified DNA of the MMP-9 promoter into the *Sac*I/*Hind*III site of the pGL2-Basic vector (29). The size of the PCR products was confirmed by electrophoresis and by DNA sequencing.

Transient transfection for MMP-9 promoter activity. Each plasmid was transfected into VSMC using SuperFect Reagent (Qiagen) according to the manufacturer's instructions (29). Luciferase activity was measured using a luciferase assay system (Promega) according to the manufacturer's instructions. Firefly luciferase activity was standardized to β -galactosidase activity.

Preparation of nuclear extracts and the electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as described elsewhere (29). Cultured cells were collected by centrifugation, washed and suspended in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM

EGTA, 1 mM DTT and 0.5 mM PMSF. After 15 min on ice, the cells were vortexed in the presence of 0.5% Nonidet P-40. The nuclear pellet was then collected by centrifugation and extracted in a buffer containing 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF for 15 min at 4°C.

The nuclear extract (10-20 μ g) was preincubated at 4°C for 30 min with a 100-fold excess of an unlabeled oligonucleotide spanning the -79 MMP-9 *cis*-element of interest. The sequences were as follows: AP-1, CTGACCCCTGA GTCAGCACTT; and, NF- κ B, CAGTGGGAATCCCCAGCC. The reaction mixture was then incubated at 4°C for 20 min in a buffer (25 mM HEPES buffer pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl and 2.5% glycerol) with 2 μ g of poly dI/dC and 5 fmol (2×10^4 cpm) of a Klenow end-labeled [³²P-ATP] 30-mer oligonucleotide, which spans the DNA binding site in the MMP-9 promoter. The reaction mixture was electrophoresed at 4°C in a 6% polyacrylamide gel using TBE (89 mM Tris, 89 mM boric acid and 1 mM EDTA) running buffer. The gel was rinsed with water, dried and exposed to X-ray film overnight.

Statistical analysis. When appropriate, data were expressed as means \pm SE. Data were analyzed by factorial ANOVA and the Fisher's least significant difference test where appropriate. Statistical significance was set at $P < 0.05$.

Results

GM3 synthase gene inhibited DNA synthesis in TNF- α -induced VSMC. To investigate the effect of the GM3 synthase gene on cell growth, VSMC were transfected with cDNA (GM3) encoding the GM3 synthase gene, or with an identical empty vector (EV) lacking a cDNA insert as a control. Established GM3 synthase gene transfectants and EV transfectants were verified by RT-PCR and immunoblot (Fig. 1A and B). The parental cells (Un) were assessed in an identical manner to serve as an additional control. We examined cell death in GM3 synthase gene and EV transfectants. As shown in Fig. 1C, the cytoplasmic DNA-histone complexes were significantly higher in the positive control than in the GM3 synthase gene- and EV-transfected cells. Next, to investigate the effect of the GM3 synthase gene on cell growth, we examined TNF- α -induced DNA synthesis in GM3 synthase gene transfectants. The [³H]-thymidine incorporation was measured for TNF- α -induced DNA synthesis. TNF- α increased DNA synthesis in EV-transfected VSMC (Fig. 2A). However, TNF- α -induced DNA synthesis was suppressed in GM3 synthase gene transfectants (Fig. 2A). This inhibition of DNA synthesis in GM3 synthase gene transfectants was recovered in a dose-dependent manner, in the presence of an anti-GM3 antibody (Fig. 2A). These data indicate that the differences observed in thymidine uptake in the GM3 synthase gene transfectants were due to cell growth inhibitory effects rather than to apoptosis.

GM3 synthase gene inhibition of ERK1/2 activation in TNF- α -induced VSMC. To elucidate how GM3 synthase gene expression suppresses TNF- α -induced cell proliferation, we examined ERK1/2 activation in EV and GM3 synthase gene

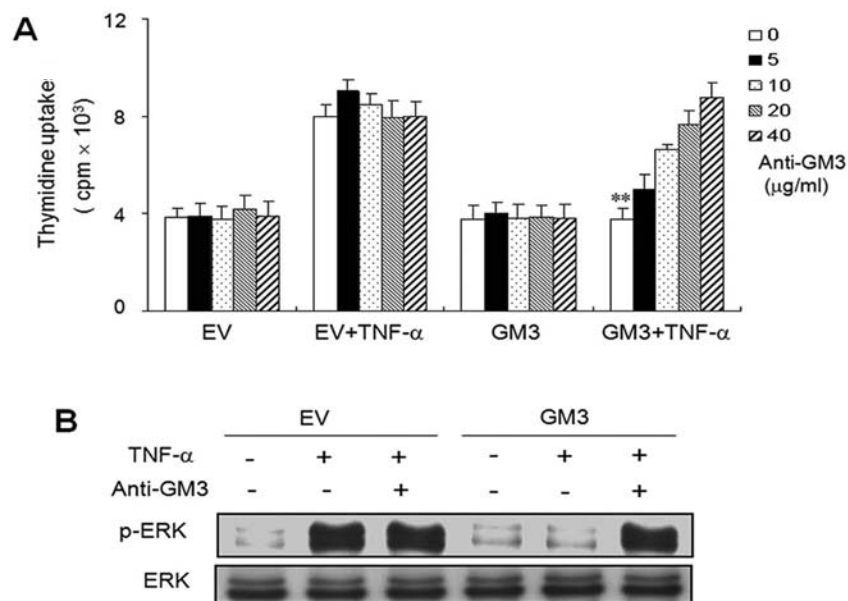


Figure 2. GM3 synthase gene expression inhibits TNF- α -induced DNA synthesis and ERK1/2 activity in VSMC. (A) Indicated cell lines were pre-cultured for 24 h in serum-free medium, stimulated with or without TNF- α (100 ng/ml) in the absence or presence of the indicated anti-GM3 antibody for 24 h. Cells were labeled with [*methyl*-³H]-thymidine during the last 24 h of this time period. Thymidine incorporation into the cells was quantified by scintillation counting of precipitated DNA. Results are presented as means \pm SE from three triplicate experiments. ** $P < 0.01$ compared with TNF- α treatment in empty vector (EV) transfectants. (B) After starvation, cell lysates from the indicated cell lines were treated with or without TNF- α (100 ng/ml) in the absence or presence of anti-GM3 antibody (40 μ g/ml) for 10 min. The cells were examined by immunoblot analysis with anti-phospho ERK1/2 antibody. The results were normalized to anti-ERK1/2 antibody expression.

transfectants. In response to TNF- α , ERK1/2 activation was stimulated in EV transfectants and suppressed in GM3 synthase gene transfectants (Fig. 2B). Moreover, the inhibited ERK1/2 activation was reversed in GM3 synthase gene transfectants after the addition of an anti-GM3 antibody to the culture medium (Fig. 2B). These results suggest that overexpression of the GM3 synthase gene may affect the TNF- α -induced inhibition of cell proliferation by decreasing ERK1/2 activation.

GM3 synthase gene attenuation of TNF- α -induced invasion and migration of VSMC. Previous studies have reported that the invasion and migration of VSMC plays an important role in the progression of atherosclerosis (8,9). We next investigated whether GM3 synthase gene expression is involved in the inhibition of invasion and migration. As shown in Fig. 3, after TNF- α treatment, the invasion and migration of VSMC increased in EV transfectants. Overexpression of the GM3 synthase gene strongly inhibited TNF- α -induced invasion and migration (Fig. 3). The inhibition of invasion and migration in GM3 synthase gene transfectants was also reversed after addition of the antibody (40 μ g/ml) for 24 h. However, addition of an anti-GM3 antibody did not affect invasion and migration in EV transfectants (Fig. 3).

GM3 synthase gene inhibition of TNF- α -induced MMP-9 expression. Recent studies have suggested that MMP-9 expression is necessary for the invasion and migration of VSMC, which are strongly associated with atherosclerosis (10-14). We next examined whether the inhibition of invasion and migration induced by GM3 synthase gene expression is

involved in the inhibition of MMP-9 expression. As shown in Fig. 4A, TNF- α stimulated MMP-9 expression in EV transfectants, as evidenced by gelatin zymography and immunoblot analysis. Overexpression of the GM3 synthase gene significantly suppressed TNF- α -induced MMP-9 secretion (Fig. 4A). Similar results were observed in the immunoblotting experiment (Fig. 4A). In cells transfected with the GM3 synthase gene, the addition of anti-GM3 antibody led to recovery of MMP-9 levels after TNF- α treatment (Fig. 4A). In contrast, MMP-9 expression by TNF- α had no effect on EV transfectants after the addition of the anti-GM3 antibody (Fig. 4A).

GM3 synthase gene expression inhibits the TNF- α -induced MMP-9 promoter by decreasing the NF- κ B and AP-1 binding activity. We further investigated how the GM3 synthase gene regulates the inhibition of MMP-9 expression. In a previous study, NF- κ B and AP-1 were identified as minimal TNF- α -response elements, which are located in the region -710 bp upstream of the transcription start site in VSMC (29,30). Therefore, we used a pGL2-MMP-9 WT plasmid (see Materials and methods for details), which spanned a luciferase reporter gene controlled by a 710-bp segment from the 5'-promoter region of the human MMP-9 gene (29,30). Both GM3 synthase gene transfectants and EV transfectants were transiently transfected with the pGL2-MMP-9 WT plasmid, and subsequently TNF- α was added for 24 h (Fig. 4B). TNF- α strongly stimulated transcriptional activity, which corresponded to the MMP-9 promoter sequence in EV transfectants (Fig. 4B). In addition, this TNF- α -stimulated MMP-9 promoter activity was reduced in GM3 synthase

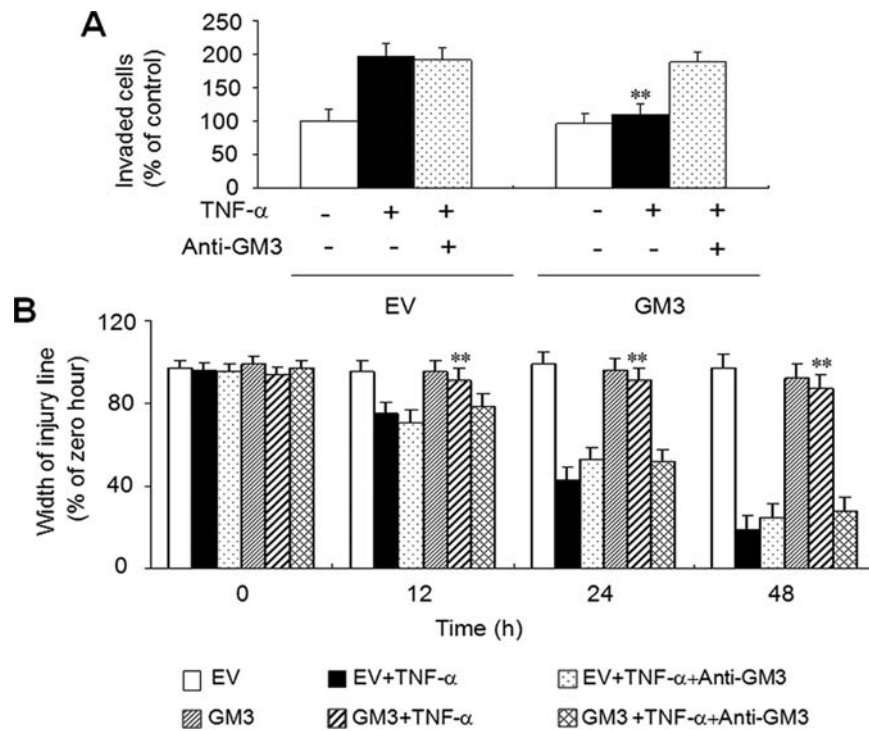


Figure 3. GM3 synthase gene expression inhibits TNF- α -induced invasion and migration of VSMC. (A) Indicated cell lines seeded in serum-free medium were placed in transwell chambers and exposed to anti-GM3 antibody (40 μ g) for 30 min before the addition of TNF- α (100 ng/ml) for 48 h. Results were analyzed for the number of invaded cells relative to the untreated control, as determined from three independent experiments. **P<0.05 compared with TNF- α treatment in empty vector (EV) transfectants. (B) The indicated cell lines in serum-free medium were exposed to anti-GM3 antibody (40 μ g) for 30 min before the addition of TNF- α (100 ng/ml). The widths of injury lines made in cells were then measured at the indicated times. Results are expressed as the widths of injury lines relative to untreated controls at 0 h, as determined from three independent experiments. **P<0.05 compared with TNF- α treatment in EV.

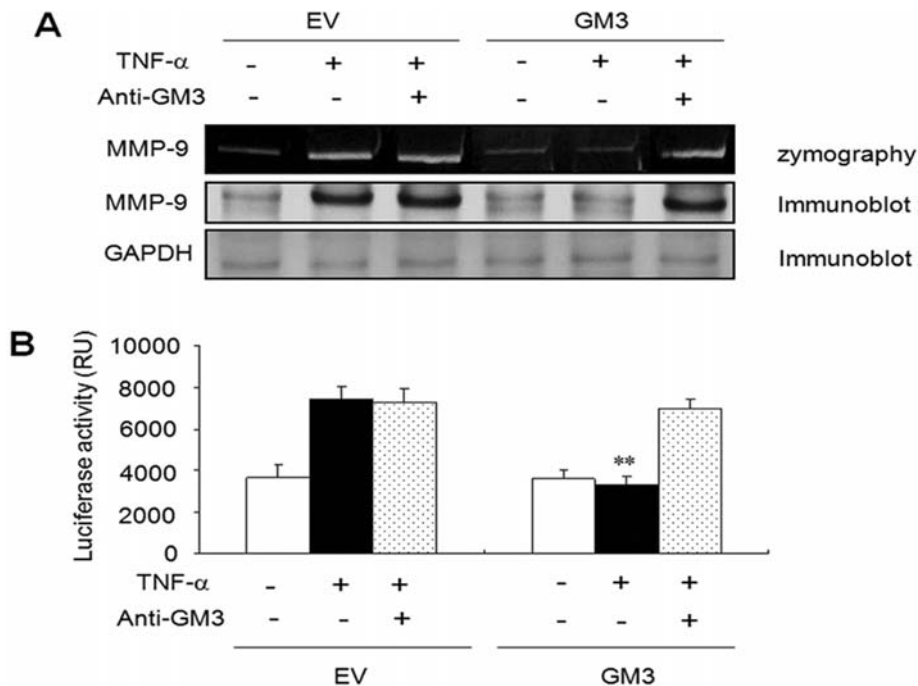


Figure 4. Overexpression of the GM3 synthase gene inhibits MMP-9 expression and its promoter activity in VSMC. (A) After a 1-day period of starvation, both the empty vector (EV) transfectants and GM3 synthase gene transfectants were pretreated with anti-GM3 antibody (40 μ g/ml) for 30 min before stimulation with or without TNF- α (100 ng/ml) for 24 h, and the gelatinolytic activities of MMP-9 were then determined from culture supernatants by a gelatin zymogram assay. Similarly, cell lysates were used for immunoblot analysis of the MMP-9 expression using an antibody specific for MMP-9. (B) The indicated cells were transiently transfected with pGL2-MMP-9 WT and then cultured with TNF- α (100 ng/ml) in the presence or absence of the anti-GM3 antibody (40 μ g/ml). Luciferase activity testing was performed to detect promoter activity of MMP-9 from cell lysates, as described in Materials and methods. **P<0.05 compared with TNF- α treatment in EV.

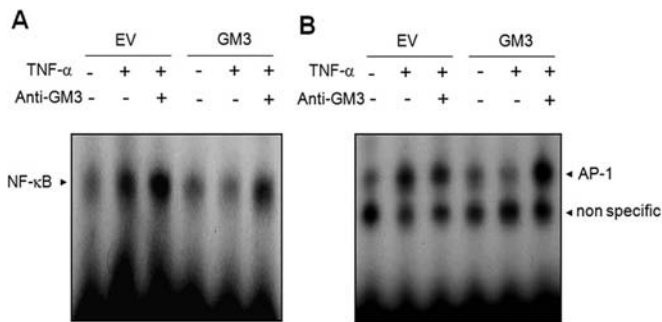


Figure 5. GM3 synthase gene expression suppresses the DNA binding activity of NF- κ B and AP-1 in TNF- α -treated VSMC. EV (empty vector) transfectants and GM3 synthase gene transfectants, pretreated with the anti-GM3 antibody (40 μ g/ml) for 40 min in serum-free medium, were incubated with TNF- α (100 ng/ml) for 24 h. After incubation, nuclear extracts from the cells were subjected to EMSA for activated (A) NF- κ B and (B) AP-1 using radiolabeled oligonucleotide probes.

gene transfectants (Fig. 4B). In the presence of an anti-GM3 antibody, the decreased MMP-9 promoter activity in GM3 synthase gene transfectants was reversed to the same levels found in EV transfectants after TNF- α treatment (Fig. 4B). These results strongly suggest that the transcription of the MMP-9 gene is involved, at least in part, in the inhibition of MMP-9 expression induced by the GM3 synthase gene.

We next used an EMSA assay to investigate whether the inhibitory effect of the GM3 synthase gene on MMP-9 expression was due to either of two types of motifs, NF- κ B or AP-1 *cis*-elements. TNF- α -induced binding to NF- κ B and AP-1 motifs was markedly increased in nuclear extracts from EV transfectants (Fig. 5). Moreover, overexpression of the GM3 synthase gene effectively suppressed any increase in NF- κ B and AP-1 binding activity (Fig. 5). Finally, as shown in Fig. 5, the results indicate that inhibition of NF- κ B and AP-1 binding activities by the GM3 synthase gene were reversed by pretreatment with an anti-GM3 antibody. These results suggest that GM3 synthase gene expression blocks TNF- α -induced MMP-9 expression by suppressing the activation of the transcription factors NF- κ B and AP-1.

Discussion

Many studies have demonstrated the ganglioside composition involved in the development and progression of atherosclerosis (23-25). Studies have shown that GM1 and GM2 induce VSMC proliferation through ERK1/2 activation (31). Several studies have shown a dual function of ganglioside GD3, in both cell proliferation and inhibition of cell growth in VSMC (32,33). A number of studies have demonstrated massive and excessive accumulation of GM3 in atherosclerotic lesions (23,24). Other studies reported that exogenously-supplied GM3 inhibited DNA synthesis independently of ERK1/2 activation in VSMC (31). Furthermore, the addition of GM3 to the culture medium reduced PDGF-induced signaling pathways in VSMC (25). Although many studies have investigated the effects of ganglioside GM3 in VSMC (23-25,31), the exact role of GM3 endogenously generated in VSMC remains unclear. In

this study, the effects of GM3 synthase gene expression on TNF- α signaling in VSMC were examined.

The first step was to analyze the ganglioside composition of VSMC, using a cloned GM3 synthase gene. TLC analysis of total gangliosides was not possible, because the growth of GM3 synthase gene transfectants was very slow. However, increased levels in the GM3 synthase gene and protein were confirmed by RT-PCR and immunoblot analyses. Although GM3 synthase gene transfectants led to a reduction in cell proliferation, there was no evidence of apoptosis in GM3 synthase gene transfected cells. Next, to understand the mechanism of cell proliferation associated with GM3 synthase gene expression, the functional role of the GM3 synthase gene in TNF- α signaling was investigated. A number of studies have identified the effects of TNF- α signaling in the formation of atherosclerotic lesions (3-7). In the present study, GM3 synthase gene transfectants showed markedly reduced thymidine uptake and ERK1/2 activation reactions in response to TNF- α compared with EV transfectants. In both EV and GM3 synthase gene transfectants, the basal levels of thymidine uptake and ERK1/2 activation remained essentially unchanged. Blocking of GM3 by the anti-GM3 antibody prevented a decrease in thymidine uptake and ERK1/2 activation induced by TNF- α . Thus, overexpression of the GM3 synthase gene showed suppressive proliferative effects on TNF- α -treated cells. In contrast with our results, previous reports showed that the addition of exogenous GM3 inhibited cell proliferation at the basal level (25,31). These results may explain the differences in the physical and functional association with the cellular membrane during cell proliferation by exogenously supplied and endogenously generated GM3 in VSMC, differences which remain to be elucidated.

It is generally accepted that disruption of the ECM is strongly controlled by the migration and invasion of VSMC, which results in the progression of atherosclerotic lesions (8,9). We further examined the effect of GM3 synthase gene expression in TNF- α -induced invasion and migration. Earlier studies demonstrated that GM3 induced inhibition of cell migration and invasion in rat glioma cells and keratinocyte-derived SCC12 cells (21,34). On the other hand, other findings indicated that GM3 stimulated the proliferation, migration, and invasion of glioma and melanoma cells (35,36). In the present study, GM3 expression by transfection with the GM3 synthase gene led to the inhibition of invasion and migration in TNF- α -induced VSMC.

Previous studies have demonstrated that MMP-9 regulation is involved in the migration and invasion of cells (10-14). Based on previous reports showing that transcription factors AP-1 and NF- κ B are essential factors in TNF- α -induced MMP-9 expression in VSMC (29), we next focused on the issue of how the expression of the GM3 synthase gene results in the inhibition of migration and invasion. TNF- α -induced MMP-9 expression was effectively suppressed by GM3 synthase gene expression. Consistent with the analyses of MMP-9, the data from the present study showed that overexpression of the GM3 synthase gene resulted in the inhibition of TNF- α -induced MMP-9 promoter activity. Finally, using consensus AP-1 and NF- κ B probes, it was found that AP-1 and NF- κ B binding activity was markedly

decreased in TNF- α -induced VSMC followed by GM3 synthase gene expression. It has recently been shown that ganglioside GM3 inhibited MMP-9 expression induced by fibronectin or epidermal growth factor (EGF) in SCC12 carcinoma cells (21). In addition, the present results are in agreement with a previous report showing the inhibition of MMP-9 expression in GD3 synthase gene transfectants (33). However, the differences in the effects of the molecular action mechanisms of the GM3 and the GD3 synthase genes remain to be elucidated. The present study is the first to show that GM3 synthase gene expression inhibits TNF- α -induced MMP-9 expression via suppressing AP-1 and NF- κ B binding activity.

This is also the first report of the inhibition of TNF- α signaling in GM3 synthase gene transfected cells. In summary, the data provide novel evidence that GM3 synthase gene expression inhibits cell proliferation via suppression of ERK1/2 activation in TNF- α -treated VSMC. In addition, the molecular mechanism underlying the GM3-mediated inhibition of migration and invasion was found to be the suppression of MMP-9 expression via decreased binding activity of AP-1 and NF- κ B. The present findings explain, in part, the beneficial possibility that the ganglioside GM3 synthase gene could prevent or treat atherosclerosis. Further studies will be required to clarify the potency of GM3 synthase gene therapy *in vivo*.

Acknowledgements

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (KRF-2008-331-C00221).

References

- Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362: 801-809, 2003.
- Abedi H and Zachary I: Signalling mechanisms in the regulation of vascular cell migration. *Cardiovasc Res* 30: 544-556, 1995.
- Jovinge S, Hultgardh-Nilsson A, Regnstrom J and Nilsson J: TNF- α activates smooth muscle cell migration in culture and is expressed in the balloon-injured rat aorta. *Arterioscler Thromb Vasc Biol* 17: 490-497, 1997.
- Tipping PG and Hancock WW: Production of tumor necrosis factor and interleukin-1 by macrophages from human atherosclerotic plaques. *Am J Pathol* 142: 1721-1728, 1993.
- Clausell N, De Lima VC and Molossi S: Expression of tumor necrosis factor- α and accumulation of fibronectin in coronary artery restenotic lesions retrieved by atherectomy. *Br Heart J* 73: 534-539, 1995.
- Rus HG, Niculescu F and Vlaicu R: Tumor necrosis factor- α in human arterial wall with atherosclerosis. *Atherosclerosis* 89: 247-254, 1991.
- Goetze S, Kintscher U, Kaneshiro K, Meehan WP, Collins A, Fleck E, Hsueh WA and Law RE: TNF α induces expression of transcription factors c-fos, Egr-1, and Ets-1 in vascular lesions through extracellular signal-regulated kinases 1/2. *Atherosclerosis* 159: 93-101, 2001.
- Schwartz SM: Perspectives series: cell adhesion in vascular biology: smooth muscle migration in atherosclerosis and restenosis. *J Clin Invest* 99: 2814-2816, 1997.
- Newby AC and Zaltsman AB: Molecular mechanisms in intimal hyperplasia. *J Pathol* 190: 300-309, 2000.
- Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Unemori EN, Lark WW, Amento E and Libby P: Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ Res* 75: 181-189, 1994.
- Fabunmi RP, Baker AH, Murray EJ, Booth RFG and Newby AC: Divergent regulation by growth factors and cytokines of 95 kDa and 72 kDa gelatinases and tissue inhibitors or metalloproteinases-1, -2, and -3 in rabbit aortic smooth muscle cells. *Biochem J* 315: 335-342, 1996.
- Cho A, Graves J and Reidy MA: Mitogen-activated protein kinases mediate matrix metalloproteinase-9 expression in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 20: 2527-2532, 2000.
- Bendeck MP, Zempo N, Clowes AW, Galaray RE and Reidy MA: Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Circ Res* 75: 539-545, 1994.
- Cho A and Reidy MA: Matrix metalloproteinase-9 is necessary for the regulation of smooth muscle cell replication and migration after arterial injury. *Circ Res* 91: 845-851, 2002.
- Huwyler A, Kolter T, Pfeilschifter J and Sandhoff K: Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochim Biophys Acta* 1485: 63-99, 2000.
- Watanabe R, Ohyama C, Aoki H, Takahashi T, Satoh M, Saito S, Hoshi S, Ishii A, Saito M and Arai Y: Ganglioside GM3 overexpression induces apoptosis and reduces malignant potential in murine bladder cancer. *Cancer Res* 62: 3850-3854, 2002.
- Hamamura K, Furukawa K, Hayashi T, Hattori T, Nakano J, Nakashima H, Okuda T, Mizutani H, Hattori H, Ueda M, Urano T, Lloyd KO and Furukawa K: Ganglioside GD3 promotes cell growth and invasion through p130Cas and paxillin in malignant melanoma cells. *Proc Natl Acad Sci USA* 102: 11041-11046, 2005.
- Kawamura S, Ohyama C, Watanabe R, Satoh M, Saito S, Hoshi S, Gasa S and Orikasa S: Glycolipid composition in bladder tumor: a crucial role of GM3 ganglioside in tumor invasion. *Int J Cancer* 94: 343-347, 2001.
- Saha S and Mohanty KC: Correlation of gangliosides GM2 and GM3 with metastatic potential to lungs of mouse B16 melanoma. *J Exp Clin Cancer Res* 22: 125-134, 2003.
- Satoh M, Ito A, Nojiri H, Handa K, Numahata K, Ohyama C, Saito S, Hoshi S and Hakomori SI: Enhanced GM3 expression, associated with decreased invasiveness, is induced by brefeldin A in bladder cancer cells. *Int J Oncol* 19: 723-731, 2001.
- Wang XQ, Sun P and Paller AS: Ganglioside GM3 inhibits matrix metalloproteinase-9 activation and disrupts its association with integrin. *J Biol Chem* 278: 25591-25599, 2003.
- Yamashita T, Hashiramoto A, Haluzik M, Mizukami H, Beck S, Norton A, Kono M, Tsuji S, Daniotti JL, Werth N, Sandhoff R, Sandhoff K and Proia RL: Enhanced insulin sensitivity in mice lacking ganglioside GM3. *Proc Natl Acad Sci USA* 100: 3445-3449, 2003.
- Bobryshev YV, Golovanova NK, Tran D, Samoilova NN, Gracheva EV, Efremov EE, Sobolev AY, Yurchenko YV, Lord RS, Cao W, Lu J, Saito M and Prokazova NV: Expression of GM3 synthase in human atherosclerotic lesions. *Atherosclerosis* 184: 63-71, 2006.
- Bobryshev YV, Lord RS, Golovanova NK, Gracheva EV, Zvezdina ND, Sadovskaya VL and Prokazova NV: Incorporation and localisation of ganglioside GM3 in human intimal atherosclerotic lesions. *Biochim Biophys Acta* 1361: 287-94, 1997.
- Sachinidis A, Kraus R, Seul C, Meyer zu Brickwedde MK, Schulte K, Ko Y, Hoppe J and Vetter H: Gangliosides GM1, GM2 and GM3 inhibit the platelet-derived growth factor-induced signalling transduction pathway in vascular smooth muscle cells by different mechanisms. *Eur J Cell Biol* 71: 79-88, 1996.
- Moon SK, Thompson LJ, Madamanchi N, Ballinger S, Papaconstantinou J, Horaist C, Runge MS and Patterson C: Aging, oxidative responses, and proliferative capacity in cultured mouse aortic smooth muscle cells. *Am J Physiol Heart Circ Physiol* 280: H2779-H2788, 2001.
- Moon SK, Jung SY, Choi YH, Lee YC, Patterson C and Kim CH: PDTTC, metal chelating compound, induces G1 phase cell cycle arrest in vascular smooth muscle cells through inducing p21Cip1 expression: involvement of p38 mitogen activated protein kinase. *J Cell Physiol* 198: 310-323, 2004.
- Oak MH, El Bedoui J, Anglard P and Schini-Kerth VB: Red wine polyphenolic compounds strongly inhibit pro-matrix metalloproteinase-2 expression and its activation in response to thrombin via direct inhibition of membrane type 1-matrix metalloproteinase in vascular smooth muscle cells. *Circulation* 110: 1861-1867, 2004.

29. Moon SK, Cha BY and Kim CH: ERK1/2 mediates TNF- α -induced matrix metalloproteinase-9 expression in human vascular smooth muscle cells via the regulation of NF- κ B and AP-1: Involvement of the ras dependent pathway. *J Cell Physiol* 198: 417-427, 2004.
30. Sato H and Seiki M: Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene* 8: 395-405, 1993.
31. Gouni-Berthold I, Seul C, Ko Y, Hescheler J and Sachinidis A: Gangliosides GM1 and GM2 induce vascular smooth muscle cell proliferation via extracellular signal-regulated kinase 1/2 pathway. *Hypertension* 38: 1030-1037, 2001.
32. Bhunia AK, Schwarzmann G and Chatterjee S: GD3 recruits reactive oxygen species to induce cell proliferation and apoptosis in human aortic smooth muscle cells. *J Biol Chem* 277: 16396-16402, 2002.
33. Moon SK, Kim HM, Lee YC and Kim CH: 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. *J Biol Chem* 279: 33063-33070, 2004.
34. Fujimoto Y, Izumoto S, Suzuki T, Kinoshita M, Kagawa N, Wada K, Hashimoto N, Maruno M, Nakatsuji Y and Yoshimine T: Ganglioside GM3 inhibits proliferation and invasion of glioma. *J Neurooncol* 71: 99-106, 2005.
35. Maidment SL, Merzak A, Koochekpour S, Rooprai HK, Rucklidge GJ and Pilkington GJ: The effect of exogenous gangliosides on matrix metalloproteinase secretion by human glioma cells in vitro. *Eur J Cancer* 32: 868-871, 1996.
36. Wang XQ, Sun P, Go L, Koti V, Fliman M and Paller AS: Ganglioside GM3 promotes carcinoma cell proliferation via urokinase plasminogen activator-induced extracellular signal-regulated kinase-independent p70S6 kinase signaling. *J Invest Dermatol* 126: 2687-2696, 2006.