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Ganglioside GD1a regulation of caveolin-1 and Stim1 expression in mouse FBJ cells: Augmented expression of caveolin-1 and Stim1 in cells with increased GD1a content

Li Wang* · Shizuka Takaku* · Pu Wang · Dan Hu · Sumiko Hyuga · Toshinori Sato · Sadako Yamagata · Tatsuya Yamagata

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Abstract GD1a was previously shown responsible for regulating cell motility, cellular adhesiveness to vitronectin, phosphorylation of c-Met and metastatic ability of mouse FBJ osteosarcoma cells. To determine the particular molecules regulated by GD1a, FBJ cells were assessed for tumor-related gene expression by semi-quantitative RT-PCR. Caveolin-1 and stromal interaction molecule 1 (Stim1) expression in FBJ-S1 cells, rich in GD1a, were found to be 6 and 4 times as much, respectively, than in FBJ-LL cells devoid of GD1a. Enhanced production of caveolin-1 in protein was confirmed by Western blotting. A low-metastatic FBJ-LL cell variant, having high GD1a expression through β1-4GalNAcT-1 (GM2/GD2 synthase) cDNA transfection (Hyuga S, *et al*, Int J Cancer 83: 685-91, 1999), showed enhanced production of caveolin-1 and Stim1 in mRNA and protein, compared to mock-transfectant M5. Incubation of FBJ-M5 cells with exogenous GD1a augmented the expression of caveolin-1 in mRNA and protein and Stim1 in mRNA as well. Treatment of FBJ-S1 with fumonisin B1, an inhibitor of *N*-acylsphinganine synthesis, for 15 days caused the complete depletion of gangliosides and suppressed the expression of caveolin-1 and Stim1. St3gal5 siRNA transfected cells showed decreased expression of caveolin-1 and Stim1 mRNA, as well as St3gal5 mRNA. These findings clearly indicate ganglioside GD1a to be involved in the regulation of the transformation suppressor genes, caveolin-1 and Stim1. Moreover, treatment with GD1a of mouse melanoma B16 cells and human hepatoma HepG2 cells brought about elevated expression of caveolin-1 and Stim1.

Keywords Caveolin-1 · Ganglioside · GD1a · Metastasis · RT-PCR · Stim-1

Abbreviations

GD1a	Neu5Acα3Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer
GD3	Neu5Acα8Neu5Acα3Galβ4GlcCer
Gg3	GalNAcβ4Galβ4 Glc
GM1	Galβ3GalNAcβ4(Neu5Acα3)-Galβ4GlcCer
GM1b	Neu5Acα3Galβ3GalNAcβ4Gal-β4GlcCer
GM3	Neu5Acα3Galβ4GlcCer
GT1b	Neu5Acα3Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4GlcCer
GM2/GD2 synthase	UDP-N-acetyl-α-D-galactosamine: (N-acetylneuraminyl)-galactosylglucosylceramide-β-1,4-N-acetylgalactosaminyltransferase
HPTLC	high performance thin layer chromatography
PCR	polymerase chain reaction

*Li Wang and Shizuka Takaku are equal contributors to the present work.

L. Wang · P. Wang · D. Hu · S. Yamagata · T. Yamagata (✉)
Laboratory of Tumor Biology and Glycobiology, Shenyang Pharmaceutical University, Shenyang 110016, People's Republic of China
e-mail: tcyamagata@opal.dti.ne.jp

S. Takaku
Department of Neurology, Dokkyo University School of Medicine, Shimotsuga, Tochigi 321-0293, Japan

S. Hyuga
Department of Clinical Research, Oriental Medicine Research Center, The Kitasato Institute, Minato-ku, Tokyo 108-8642, Japan

T. Sato
Department of Biosciences and Informatics, Keio University, Hiyoshi, Yokohama 223-8522, Japan

D-PDMP	D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
St3gal5	CMP-NeuAc:lactosylceramide alpha-2,3-sialyltransferase

Introduction

Ganglioside GD1a regulates cell motility, cell adhesiveness to vitronectin, phosporylation of c-Met and metastatic capacity of mouse FBJ cell lines [1–3]. Using cells derived from FBJ-virus-induced mouse osteosarcoma, FBJ-S1 cells with no indication of metastasis were found to express gangliosides GM3 and GD1a, whereas FBJ-LL cells, capable of mestasizing to lung and liver subsequent to subcutaneous transplantation of cells to mouse thigh, possessed GM3 and GD1a but the latter in only limited amounts. FBJ-LL cells showed greater cell motility in a Transwell experiment and stronger adhesiveness to vitronectin compared to FBJ-S1 cells. Pretreatment of FBJ-LL cells with GD1a suppressed cell motility and reduced cell adhesiveness to vitronectin as FBJ-S1 cells. Transfection of GM2/GD2 synthase cDNA to FBJ-LL cells gave rise to several transfectants with GD1a expressed to varying degrees and LA5-22 and LA5-30, each with the same degree of GD1a expression as FBJ-S1, were found to have essentially the same cell motility and cell adhesiveness to vitronectin as FBJ-S1 cells. LA5-22 cells showed no indication of metastasis in mice to whose thighs these cells had been administered and which were sacrificed at 4 to 5 weeks to confirm the presence of tumor cells in organs macro- and microscopically. M5 cells as a control mock transfectant were found to have metastasized to lung, liver and kidney [2]. GD1a is thus shown to control the metastatic machinery of FBJ cells but the underlying mechanism for this remains to be clarified.

Caveolin was identified as a phosphorylated protein in the tyrosyl residue in Rous sarcoma virus transfected avian fibroblasts [4–7] and has been shown a major molecular constituent in caveolae [8]. Caveolin-2 [9–11] and caveolin-3 [12,13], other family members, have been reported. Caveolins-1 and -2 [9–11] have been shown to constitute heterooligomers and to be expressed in numerous cell types though not lymphocytes. Caveolin-3 [12,13] is specifically expressed in muscle cells. Caveolin-1 has been found indispensable to the formation of caveolae by functioning as a scaffolding protein to recruite and organize lipid-modified signal transducing molecules, cholesterol and glycosphingolipid [14–18]. Caveolae on plasma membranes are membrane domains that include lipid rafts where signal transducing molecules such as Src family tyrosin kinases, the EGF receptor and heterotrimeric G proteins condense [19,20]. Via its scaffolding domain, caveolin-1 interacts with signal molecules and regulates their activity [19]. Caveolin-1 is anchored to the actin cytoskeleton, particularly the actin arc [21] and quite possibly has involvement in transducing exogenous signals inside cells. There is considerable evidence that caveolin-1 serves as a transformation suppressor protein [19,22]. Caveolin-1 has been down-regulated in cells transfected with oncogene [23,24] and its expression suppressed in human and mouse tumor cells [25]. Its overexpression in human mammary cancer cells inhibits cell proliferation [26]. Transgenic mice administered with MMTV polyoma virus develop mammary tumors, while caveolin-1 knockout mice derived from transgenic mice show an accelerated onset of mammary tumors and tumor metastasis to the lung [27]. Caveolin-1 expression in cells metastasized in the lung diminishes cell invasion into Matrigel and the activity of MMP-9 and MMP-2. Caveolin-1 is thus shown to have central involvement in metastasis.

Stim1 is mapped at human chromosome 11p15.5; its position is related to tumorigenesis and this gene is regarded as a tumor suppressor gene [28]. Stim1 is an integral membrane *N*-glycosylated glycoprotein consisting of 746 amino acid residues [29,30] with 90% sequence identity in human and mouse proteins [31]. Using rhabdomyosarcoma cells *in vitro* and rhabdoid tumors *in vivo*, Stim1 was shown to inhibit the growth of tumor cells [32] and be expressed in many human primary and transformed cell lines [30]. The selective suppression of stim1 mRNA brought about rhabdomiasarcoma tumorigenesis [32] and with the application of randomized ribozymes to metastasizing cells, Stim1 mRNA broke down to promote the pulmonary metastasis of B16F0 cells [33]. Stim1 and Stim2 have recently been shown essential to Ca^{2+}-store-depletion-mediated Ca^{2+}-influx or store-operated Ca^{2+} (SOC) influx, based on data obtained using siRNA techniques [34,35].

The present authors previously noted an inverse relationship between GD1a and metastatic capability of FBJ cells and thus examination was made as to whether the expression of caveolin-1, a tumor suppressor protein, may be under the control of GD1a. Highly metastatic FBJ-LL and mock transfectant M5 cells were found to express less caveolin-1 and Stim1 compared to non-metastatic FBJ-S1 and GD1a-expressing transfectant LA5-30 cells. Treatment of M5 cells with GD1a enhanced caveolin-1 expression in mRNA and protein and also Stim1 mRNA. Fumonisin B1 treatment of FBJ-S1 cells caused the complete depletion of GD1a, with consequent decrease in the expression of caveolin-1 and Stim1. St3gal5 siRNA transfected cells suppressed the expression of caveolin-1 and Stim1 mRNA as well as that of St3gal5 mRNA. GD1a treatment of mouse B16 melanoma and human HepG2 cells increased caveolin-1 and Stim1 expression, suggesting GD1a to have effect for regulating these genes under any and all circumstances. From these findings, GD1a is clearly shown to regulate the expression of caveolin-1 and Stim1, both possibly involved in the transformation suppressor genes.

Materials and methods

Cell lines and culture

The highly metastatic mouse osteosarcoma cell line, FBJ-LL, and poorly metastatic cell line, FBJ-S1, were produced from a FBJ virus-induced osteosarcoma of the BALB/c mouse [36,37]. FBJ-S1 cells expressed GM3 and GD1a, whereas FBJ-LL cells expressed GM3 and slightly expressed GD1a [1]. The capacity for FBJ-LL cells to migrate was ten times that of FBS-S1 cells, but decreased by a half by treatment with GD1a [1]. FBJ-LL cells mestasized into the liver and lung, but FBJ-S1 did not [36]. LA5-22, LA5-30 cells were obtained by transfection of FBJ-LL cells with β1-4GalNAcT-1 (GM2/GD2-synthase), and mock-transfectant M5 cells, as control [3]. GD1a expression in LA5-22 and LA5-30 cells was 5-fold that of M5. Migration capacity of LA5-22, LA5-30 cells was about one tenth that of M5, comparable to the capacity of FBJ-S1 cells. When M5 cells were inoculated into mice, metastatic nodules were observed in liver, lung, kidney and adrernal glands within 4 to 5 weeks, while LA5-22 cell transplantation did not show any sign of metastasis [3].

Mouse melanoma B16 and human HepG2 cells were kindly provided by Dr. Kiyoshi Furukawa at Nagaoka Univeristy of Technology, Japan. The cells were maintained in medium containing RPMI-1640 (GIBICO, Invitrogen Corporation, N. Y., U.S.A.) supplemented with 10% fetal bovine serum (TBD-TianJin HaoYang Biological Company, TianJin, China), 100 units/ml penicillin, and 100 μg/ml streptomycin, and incubated in a humidified (37 °C, 5% CO_2 and 95% air) incubator (Sanyo, Tokyo, Japan). The cells were usually grown in a 60 mm culture dish (BD Falcon, CA, U.S.A.) and passaged on reaching 75% confluency. For ganglioside treatment, the cells were seeded, washed with serum free RPMI-1640 at 24 h, incubated with ganglioside at the specified concentration in the absence of serum for 4 h, supplemented with serum to the final 5% concentration and further cultivated for an additional 20 h in serum-containing medium. Procedure repetition was done as required. To suppress glycolipid synthesis, the cells were cultivated in the presence of D-PDMP, L-PDMP or fumonisin B1 at a specified concentration and provided fresh medium containing the inhibitor every three days.

Chemicals

Ganglioside GD1a from bovine brain was purchased from Wako (Osaka, Japan). Gangliosides GM1, GD1b and GT1b from bovine brain were purchased from Toyobo (Osaka, Japan), ganglioside GD3, disodium salt from bovine brain was from Alexis (U.S.A.). The primers used in this study were designed by Primer3 software and synthesized by Shanghai Genebase Biotechnology Corporation. Rabbit anti-caveolin-1 polyclonal antibody and mouse anti-actin polyclonal antibody were from Santa Cruz Biotechnology, Inc. (CA, U.S.A.). Horseradish peroxidase (HRP)-linked anti-mouse IgG secondary antibody and anti-rabbit IgG secondary antibody were from Cell Signaling (MA, U.S.A.). The RNeasy Mini Kit to extract total RNA was purchased from QIAGEN (Hilden, Genmany). The RT-PCR kit was from the TAKARA Biotechnology Corporation (Dalian, China) and D-PDMP, L-PDMP and fumonisin B1, from Sigma.

RNA extraction and RT-PCR

2×10^6 cells were harvested and total RNA was extracted using the QIAGEN RNEasy Kit according to the instructions of manufacturer. 1 μg RNA, taken as indicated by absorption, was subjected to RT-PCR using the TaKaRa RT-PCR kit (AMV) Ver. 3.0 with a PC707 Program Temp Cont System (ASTEC, Japan). The product obtained was analyzed by 2% agarose electrophoresis. Following ethidium bromide staining (0.05% ethidium bromide in TAE buffer) for 30 min, the intensity of the stained band was assessed with a Bio-profile Bio 1D image analyzer (Vilber Lourmat, Marne-la-Vallee, France) at 312 nm. Primers were synthesized at the GeneBase (Shanghai, China) and primer sequences used for PCR in this study were as follows: for Actb (β-actin), Sense 5′-ACACTGTGTGCCCATCTACGAGG-3′ and Antisense 5′-AGGGGCCGGACTCGTCGTCATACT-3′; for caveolin-1 (Cav1), Sense 5′-CTACAAGCCCAACAACAAGGC-3′ and Antisense 5′-AGGAAGCTCTTGATGCACGGT-3′; for stromal interaction molecule-1 (Stim1), Sense 5′-TGCTGTTTGGGCCTCCTCTC-3′ and Antisense 5′-CCTCCACCTCATGGGTCAGC-3′; for Itgb5 (integrin β5), Sense, 5′-TGGCCAGTGTCACCTGAATGA-3′ and Antisense 5′-GCCGCCTCAAGGTGAAAGACT-3′; for Tgfb1 (TGFβ1), Sense 5′-CCAGCGACATGGAGCTGGTG-3′ and Antisense 5′-AGGCGTATCAGTGGGGGTCA-3′; for Tgfbr2 (TGFβR2), Sense 5′-ACGACCCAAGCTCACCTACC-3′ and Antisense 5′-GCTCCGTGTTGTGGTTGATGTT-3′; for Catna1 (cateninα1), Sense 5′-TGGAAGCCACCAAGCTCCTC-3′ and Antisense 5′-GCTTGGCCAGCACAATGATG-3′; for Rab7 (Ras-related protein Rab-7), Sense 5′-CTGGGGGACTCTGGTGTTGG-3′ and Antisense 5′-CTGGAAGGCCTGCTCCACAT-3′; for Cdh1 (E-cadherin), Sense 5′-TCCCATCCCAGAACCTCGAA-3′ and Antisense 5′-CCGCCTTCATGCAGTTGTTG-3′; for Cdh3 (P-cadherin), Sense 5′-GGGCTGGCTGTTGTTGCATA-3′ and Antisense 5′-CGCTAATGGTTCCCGTGCTT-3′; for Cdh6 (K-cadherin), Sense 5′-GCGTTGCTCAACATGGATCG-3′ and Antisense 5′-TGGGTTTCCTGGTCGGTGAT-3′;

for Icam1 (I-CAM-1), Sense 5′-TGCGTTTTGGAGCTAGCGGACCA-3′ and Antisense 5′-CGAGGACCATACAGCACGTGCAG-3′; for Ncam1 (N-CAM), Sense 5′-GATCAGGGGCATCAAGAAAA-3′ and Antisense 5′-GGAGGCTTCACAGGTCAGAG-3′; for AW551984, Sense 5′-GAGTTTGCCAATGGGCTGCT-3′ and Antisense 5′-GGGAGGCAAATTCCAGCTCA-3′; for Twist1, Sense 5′-CTGGCCTCGGACAAGCTGAG-3′ and Antisense 5′-CTCTGTCCACGGGCCTGTCT-3′; for St3gal5, Sense 5′-GCTCAAGGACCTCCCTGCAA-3′ and Antisense 5′-CGGGCAGCATATCCAAGAGG -3′.

Semi-quantitative PCR

mRNA of the genes under consideration and ß-actin mRNA as control were determined by RT-PCR semi-quantitatively, since no real-time PCR apparatus was available. DNA was noted to increase with PCR, but at a rate never reaching 2, the theoretical value. The rate was constant up to reaction plateauing, owing to depletion of substrates and/or primers, inactivation of the enzyme and other factors. No reliable results could be obtained when measurement was made using the cycle outside the linear range. PCR on the sample and Actb (ß-actin) was thus conducted at consecutive cycle numbers and DNA content was plotted on semi-log graph paper as a function of cycle number. When these parameters were linear and parallel, slope values were considered to represent actual mRNA content in a specimen. Tgfbr2 (TGFßR2) from 25 to 35 cycles, starting from 1 μg of RNA of FBJ-S1cells and Actb, from 14 to 24 cycles, are presented in Fig. 10. The two regression lines were parallel so that any content values from 27 to 31 cycles for determining TGFßR2 and 14 to 24 cycles for Actb could be used. The cycle number 16 was used for determining Actb and 25 for Tgfbr2. DNA content was divided by that of Actb and usually expressed as 1 for control experiments. This manner of determination was concluded to always provide reliable results for all specimens.

Western blotting

1×10^6 cells were lysed in 1 ml sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerine, 5% ß-mercaptoethanol, 0.01% bromophenol blue) at 37 °C for 30 min and boiled at 100 °C for 5 min. An aliquot of the lysate was loaded onto a 12.5% SDS-polyacrylamide gel. Following electrophoresis, the gel was blotted and subjected to Western blotting. The blotted membrane was reacted with rabbit anti-caveolin-1 polyclonal antibody at 1/2000 dilution, followed by horseradish peroxidase (HRP)-conjugate anti-rabbit IgG secondary antibody (1/2000 dilution). The membrane was also reacted with antibody raised against Actb followed by the second antibody to obtain the internal standard. Western blots were visualized by ECL Western blotting detection reagents (Amersham Biosciences) so as to enhance chemiluminescence subsequently to be exposed to Fuji XR film. Lanes were scanned and the optical density was determined by the Bio-profile Bio 1D image analyzer (Vilber Lourmat, Marne-la-Vallee, France).

SiRNA

Target sequences and the scrambled sequence of St3gal5 encoding mouse ganglioside GM3 synthase (Sialyltransferase 9) were selected using a Genscript program, Mulfold software and the assistance of Dr. N. Ota of Riken (Yokohama, Japan). The sequences were made to constitute a retroviral vector with neomycin resistance at TAKARA Biotechnology Corporation. Plasmids were transfected into FBJ-S1 cells in the presence of Fugene (Roche, U.S.A.) as specified by the manufacturer. At three days following transfection, RNA was extracted and assayed for expression of St3gal5, caveolin-1 and Stim1. The most effective target was found to be siRNA sequence 2, 5′-AGACGGCTATGGCTCTGTTAT-3′, followed by siRNA sequence 1, 5′-CAGGCTTTGGCTACGACCTCA-3′. The control siRNA contained the sequence 5′-TCGCGCTCCACATGATGACTA-3′.

Ganglioside extraction and HPTLC

Glycolipids were extracted from 2×10^6 cells with 1 ml chloroform/methanol 1:1 (v/v), followed by second extraction in 1 ml chloroform/methanol 2:1 (v/v). The extracts were evaporated to dryness and HPTLC analysis was conducted on a silicagel HPTLC plate (Merck, Darmstadt, Germany) using the solvent system: chloroform/methanol/0.25% aqueous KCl (5:4:1 by volume). Gangliosides were visualized by spraying resorcinol-HCl reagent followed by heating at 105°C for 20 min and the amount of the gangliosides was assessed by the Bio-profile Bio 1D image analyzer.

Results

Caveolin-1 and Stim1 expression in FBJ-S1 and LA5-30 cells far exceeding that in FBJ-LL or M5 cells

Metastatic capacity of the mouse osteosarcoma cell line was previously shown under the control of GD1a, but as to how signals attributable to GD1a are transduced to cells and mechanisms for the suppression of metastasis are all matters awaiting clarification. To elucidate the signaling pathways controlled by GD1a in relation to metastasis and the tumor-related molecules involved in these pathways, tumor suppression-related genes such as Ncam1 (N-CAM), Itgb5 (integrin β5), Tgfb1 (TGF β1), Tgfbr2 (TGF βR2), Catna1 (catenin α1), Rab7 (Ras-related protein Rab-7), Twist1 (twist gene homologue 1 Drosophila), Icam1 (I-CAM-1),

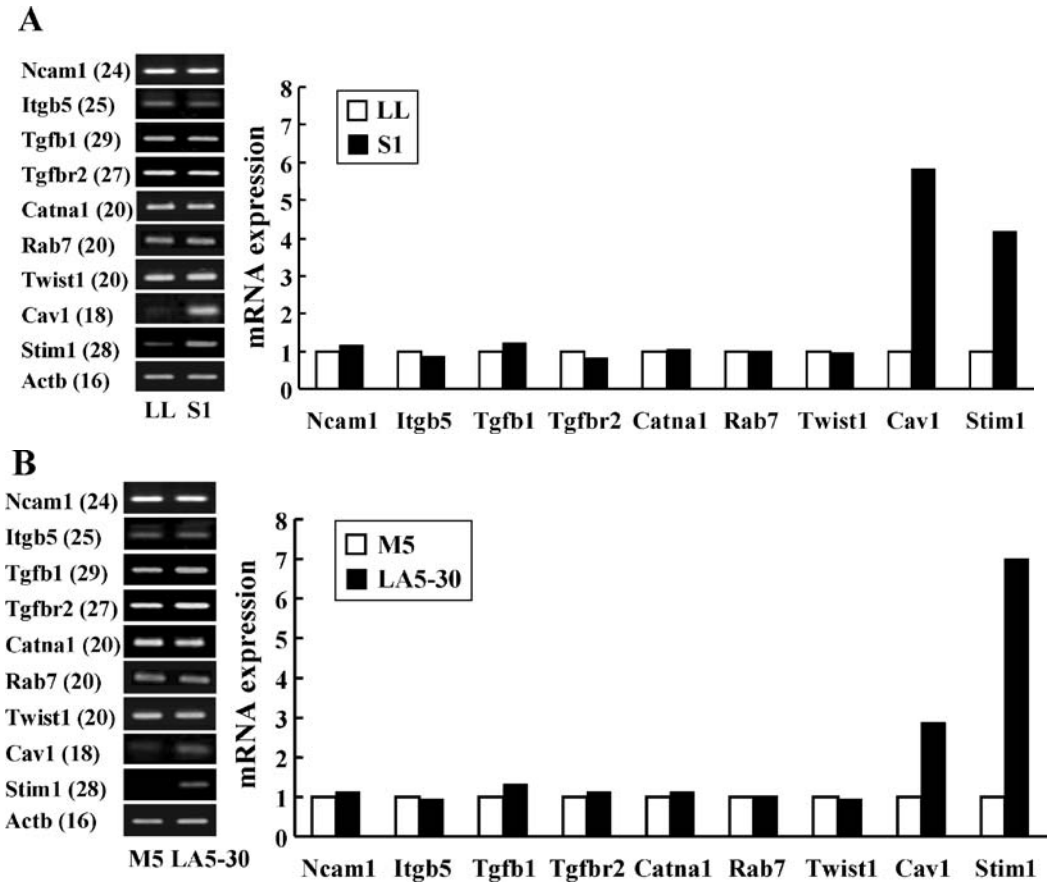

Fig. 1 Caveolin-1 and Stim1 more highly expressed in FBJ-S1 and LA5-30 cells than in FBJ-LL and M5 cells. Cells were harvested at end of the logarithmic growth phase and total RNA was extracted. Expression of Ncam1 (N-CAM), Itgb5 (integrin $\beta 5$), Tgfb1 (TGF $\beta 1$), Tgfbr2 (TGF βR2), Catna1 (catenin $\alpha 1$), Rab7 (Ras-related protein Rab-7), Twist1 (twist gene homologue 1 Drosophila), Cav1 (caveolin-1), Stim1 (stromal interaction molecule 1), and Actb (β-actin) was determined by RT-PCR in the thermal cycle in which increase in amplified DNA was proportional to the cycle. Densitometric analysis of mRNA expression in FBJ-S1 compared to FBJ-LL cells (***A***) and mRNA expression in FBJ-LA5-30 compared to FBJ-M5 cells (***B***). In Fig. 1A ordinate represents the expression ratio of various molecules in FBJ-S1 cells as compared to FBJ-LL cells at the same thermal cycle, where the ratio of DNA of gene under consideration to Actb in FBJ-LL cells is expressed as 1 and compared to the ratio of DNA of gene under consideration to Actb in FBJ-S1 cells. In Fig. 1B, the ordinate indicates relative value of expression of each molecule normalized by Actb expression as 1.0 in M5 *vs* β1-4GalNAcT-1 (GM2/GD2 synthase) transfectant LA5-30 at defined thermal cycle shown in parentheses. Each caveolin-1 and Stim1 value represents the mean of two independent determinations. Left panels show representative PCR results with the cycle number used in parentheses.

Cdh1 (E-cadherin), Cdh6 (K-cadherin), Cdh3 (P-cadherin), AW551984, Cav1 (caveolin-1) and Stim1 (stromal interaction molecule 1) were examined for their mRNA in FBJ cell lines by semi-quantitative RT-PCR. Figure 1A shows LL and S1 cell lines to be essentially the same in Ncam1, Itgb5, Tgfb1, Tgfbr2, Catna1, Rab7 and Twist1 expression, whereas that of caveolin-1 and Stim1 in S1 cells to be 6 and 4.2 times higher, respectively, the expression in FBJ-LL cells, the difference thus being significant. Icam1, Cdh1, Cdh6, Cdh3 and AW551984 expression could be found in neither cell line (data not shown). In PCR cycles, expression for Icam1, Cdh1, Cdh6, Cdh3 and AW551984 was negative at 40, 40, 30, 35 and 35, respectively. These primers showed positive bands in kidney cell extracts taken from 4-week old BALB/C female mice, thus indicating the absence of mRNA in the samples not to be due to the primers.

In a previous study, transfectants producing GD1a to the same extent as FBJ-S1 cells following transfection with GM2/GD2 synthase cDNA to FBJ-LL cells were obtained [3]. A comparison of LA5-22 and LA5-30 cells with mock transfectant M5 indicated these genes to be expressed to basically the same extent except for caveolin-1 and Stim1 (Fig. 1B, results for LA5-22 the same as for LA5-30 and thus omitted). Caveolin-1 and Stim1 expression in LA5-30 cells was 3 and 7 times higher than in FBJ-M5, respectively (Fig. 1B). Increased GD1a is thus shown clearly associated with enhanced expression of caveolin-1 and Stim1.

In addition to differences in mRNA content, Western blotting indicated the expression of caveolin-1 in FBJ-S1 and LA5-30 cells to be 7.5 times as much that in LL and M5 cellular protein (Figs. 2A and B). Stim1 did not stain for protein expression, since no antibody toward Stim1 was available.

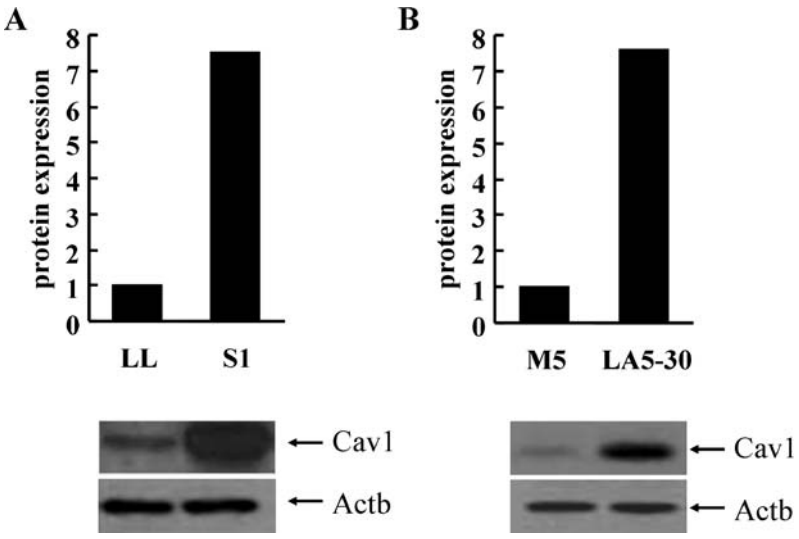

Fig. 2 Caveolin-1 protein more highly expressed in FBJ-S1 and LA5-30 cells than FBJ-LL and M5 cells. Cells were harvested at end of the logarithmic growth phase and the cell lysate was subjected to Western blotting. Densitometric analysis of caveolin-1 expression in FBJ-S1 compared to FBJ-LL cells (***A***) and that in FBJ-LA5-30 compared to FBJ-M5 cells (***B***). Ordinate represents protein in arbitrary unit where the ratio of caveolin-1 to Actb of the control is expressed as 1. Lower panels show the immunoblots.

Cells abundant in GD1a are thus far richer in caveolin-1 mRNA and protein expression and in Stim1 mRNA expression, compared to cells with lesser GD1a content. Caveolin-1 and Stim1 expression may thus reasonably be considered due to GD1a. Accordingly, examination was made as to whether GD1a regulates caveolin-1 and Stim1 expression in FBJ cell lines.

Increased caveolin-1 and Stim1 expression in M5 and LL cells by GD1a treatment

To confirm GD1a as responsible for the positive regulation of caveolin-1 and Stim1 expression, M5 and LL cells with little caveolin-1 and Stim1 expression were treated with GD1a at different concentrations for 4 h followed by incubation in medium containing serum. Treatment in the absence of serum followed by incubation for 20 h in the presence of serum was carried out two times on M5 cells, with consequent dose-dependent increase in caveolin-1 (Fig. 3A). Caveolin-1 mRNA increased by 1.5 times as much with 50 μM and 2.2 times as much with 100 μM GD1a. Increase was evident even at one day of treatment. 50 μM and 100 μM GD1a caused expression to increase caveolin-1 mRNA by 1.2 and 1.5 times as much, respectively (Fig. 3B). 1.6 increase in Stim-1 mRNA was noted with 50 μM GD1a and 1.9 increase with 100 μM GD1a in M5 cells at two days (Fig. 3A). One day GD1a treatment increased Stim1 expression in M5 cells; 50 μM and 100 μM GD1a caused expression to increase by 1.2 and 1.4 times as much, respectively. The effects of incubation with GD1a for 4 h on caveolin-1 expression were not restricted to M5 cells. Figure 3C indicates that in LL cells treated with 50 μM and 100 μM GD1a for 4 h followed by 20 h incubation with serum, caveolin-1 mRNA expression increased in a dose dependent manner; caveolin-1 expression was enhanced dose dependently by 1.3 and 1.5 times as much, respectively. This treatment had no effect on Stim1 synthesis so that GD1a regulation of Stim1 synthesis may differ from that of caveolin-1.

Exogenous GD1a addition increased caveolin-1 mRNA in M5 and LL cells as shown by RT-PCR and thus, change in the protein expression was examined by treating M5 cells and LL cells with GD1a at different concentrations for 2 days and by Western blotting using the anti-caveolin-1 polyclonal antibody. GD1a at 50 μM and 100 μM was shown to significantly increase protein expression of caveolin-1 by 1.7 and 2.4 times, respectively, and in LL cells, 1.6 and 2.1 times, respectively, that of control cells not been treated with GD1a (Figs. 4A and B). These findings are consistent with those by RT-PCR, further supporting the notion that ganglioside GD1a regulates caveolin-1 and Stim1 expression.

Increase in caveolin-1 and Stim1 mRNA by GD1a stimulation of mouse B16 and human HepG2 cells

Exogenous GD1a addition was shown to augment caveolin-1 and Stim1 synthesis in FBJ cells, indicating GD1 to be a possibly the responsible factor for this synthesis. It would follow then that GD1a may exert a similar effect on other cell lines devoid of GD1a. The mouse melanoma cell line B16 and human hepatocarcinoma HepG2 possess GM3 as the sole ganglioside species [2,38, see also Fig. 9]. B16 cells were treated with 25 μM GD1a in the same manner as M5 cells for two days, with consequent increase in caveolin-1 and Stim1 mRNA by 1.7 and 1.8 times as much, respectively (Fig. 5A). Western blotting of caveolin-1 in B16 cells treated with GD1a failed to stain, owing to very slight protein expression. Human HepG2 cells treated with 50 μM GD1a as with M5 cells and incubated for two days exhibited enhanced caveolin-1 and Stim1 expression by 1.5 times the original value (Fig. 5B). Such increase was noted not only for FBJ cells but other lines possessing no GD1a, suggesting GD1a to consistently enhance the expression of these genes.

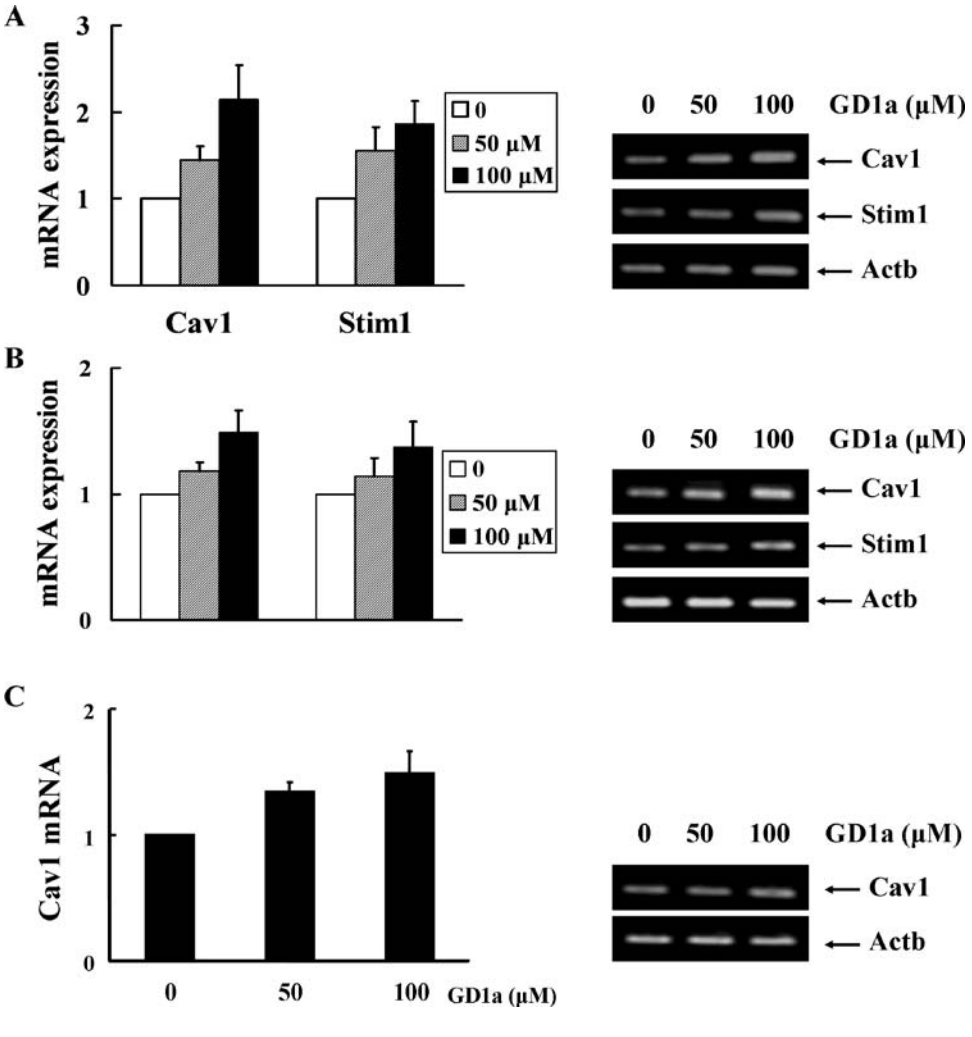

Fig. 3 Increase by GD1a treatment in caveolin-1 and Stim1 in M5 and LL mRNA. Cells were cultivated in the presence of GD1a as described in "Materials and Methods." Densitometric analysis of mRNA expression in FBJ-M5 incubated for two days (***A***) and one day (***B***) as a function of GD1a concentration. ***C***, Densitometric analysis of caveolin-1 mRNA expression in FBJ-LL incubated for two days as a function of GD1a concentration. Ordinate represents mRNA in arbitrary unit where the ratio of the amount of DNA of gene of issue to Actb of the control cells incubated without GD1a is expressed as 1. Each value represents the mean plus minus S.E. of three independent determinations. Right panels show representative PCR results.

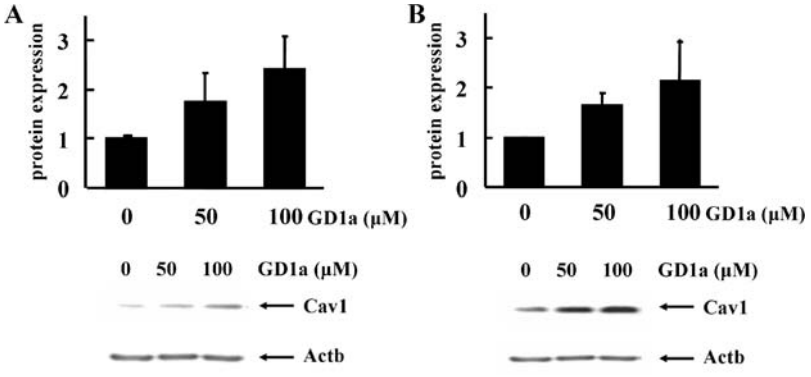

Fig. 4 Western blots showing GD1a treatment to increase caveolin-1 in M5 and LL cells. Cells were cultivated in the presence of GD1a as detailed in "Materials and Methods" and the cell lysate was subjected to Western blotting. Densitometric analysis of caveolin-1 expression in FBJ-M5 (***A***) and FBJ-LL cells (***B***) incubated for two days as a function of GD1a concentration. Ordinate represents protein in arbitrary unit where the ratio of caveolin-1 to Actb of the control is expressed as 1. Each value represents the mean of three independent determinations. Lower panels show the immunoblots.

Effects of other gangliosides on caveolin-1 and Stim1 expression in M5 cells

GD1a remarkably enhanced caveolin-1 and Stim1 expression in B16 and HepG2 as well as FBJ-M5 and FBJ-LL. Then possibly, gangliosides other than GD1a may exert a similar effect. To confirm this in the case of caveolin-1 and Stim1 expression, M5 cells were treated with the gangliosides, GM1, GD3, GD1b or GT1b at 50 μM for 2 days under the same conditions as for GD1a. As shown in Fig. 6, GM1,

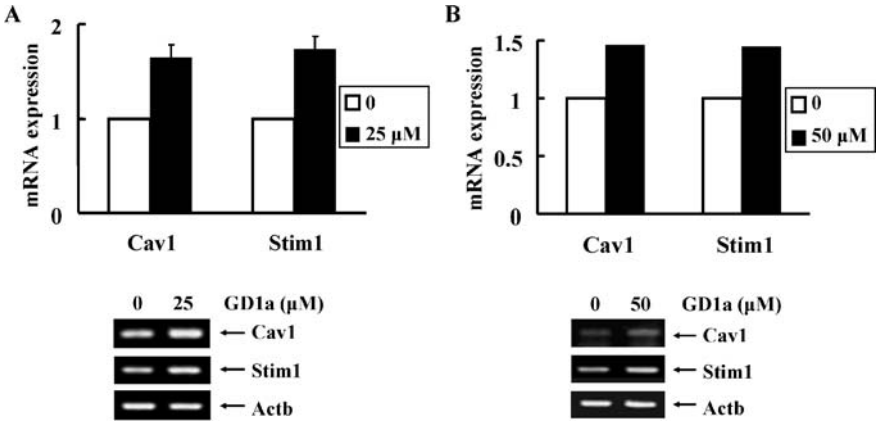

Fig. 5 Increase in caveolin-1 and Stim1 mRNA in Mouse B16 and human HepG2 cells in response to GD1a. Cells were cultivated in the presence of GD1a as detailed in "Materials and Methods." Densitometric analysis of mRNA expression in mouse melanoma B16 cells (***A***) and human HepG2 cells (***B***) incubated with GD1a for two days. Ordinate represents mRNA in arbitrary units where the ratio DNA of gene under consideration to Actb of control cells incubated without GD1a is expressed as 1. Each value for B16 cells represents the mean plus minus S.E. of three independent determinations. Lower panels show representative PCR results.

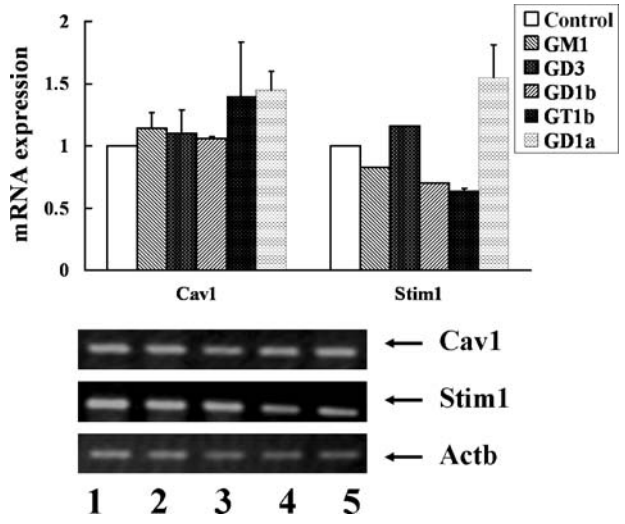

Fig. 6 Effects of other gangliosides on expression of caveolin-1 and Stim1. M5 cells were incubated with 50 μM GM1, GD3, GD1b or GT1b as described in "Materials and Methods." Densitometric analysis of mRNA expression is shown. Ordinate represents mRNA in arbitrary unit where the ratio of DNA of Cav1 or Stim1 to Actb of control cells incubated without gangliosides is expressed as 1. Each value for caveolin-1 represents the mean plus minus S.E. of three independent determinations. Value for Stim1 of cells treated with GT1b is the mean of two independent determinations. Lower panels show representative PCR results. Lane 1 represents control; lane 2, GM1; lane 3, GD3; lane 4, GD1b; lane 5, GT1b.

GD3 and GD1b had no effect on caveolin-1 but GT1b enhanced caveolin-1 expression by a factor of 1.5. GM1 and GD3 had no effect on Stim1 expression, while GD1b and GT1b suppressed Stim1 to 0.7 and 0.6 of the original value, respectively. Enhanced expression of caveolin-1 and Stim1 would thus appear to be GD1a-specific.

Inhibition of GD1a-mediated expression of caveolin-1 and Stim1

From the above, GD1a would appear quite likely to induce caveolin-1 and Stim1 expression, so that down-regulation of the synthesis of caveolin-1 and Stim1 may reasonably be considered to occur by removal of GD1a. Should impaired GD1a synthesis and depletion of GD1a serve to suppress caveolin-1 and/or Stim1, this would certainly be an indication that GD1a regulates caveolin-1 and Stim1 expression. Consequently, certain inhibitors were examined for capacity to reduce GD1a. D-PDMP blocks the synthesis of glucosyl-ceramide with consequent depletion of all glycosphingolipids such as GD1a in cells cultivated in this compound for several days. FBJ-S1 cells treated with 7.5 μM D-PDMP for 3 days failed to reduce caveolin-1 in FBJ-S1 cells though cell morphological change was noted and cell proliferation was suppressed. 12.5 μM D-PDMP inhibited caveolin-1 and Stim1 by half, this being associated with decrease in GD1a, though significant change in cell morphology occurred and proliferation appeared to cease, possibly owing to toxicity of the drug. Thus, whether reduction in GD1a from GD1a-enriched cells such as FBJ-S1 cells actually decreases caveolin-1 expression by D-PDMP remains to be fully confirmed. FBJ-S1 cells treated with fumonisin B1, an inhibitor of *N*-acylsphinganine synthesis, showed decrease in GD1a. Cell proliferation was profuse in the presence of 30 μM fumonisin B1 at 15 days incubation and RNA and lipid fractions were extracted for analysis. Figure 7A shows the expression of GD1a to have been eliminated in cells cultivated with 30 μM fumonisin B1 for 15 days and in Fig. 7B, caveolin-1 and Stim1 mRNA expression can be seen inhibited by 42% and 68%, respectively, thus showing GD1a involvement in the regulation of caveolin-1 and Stim1 synthesis.

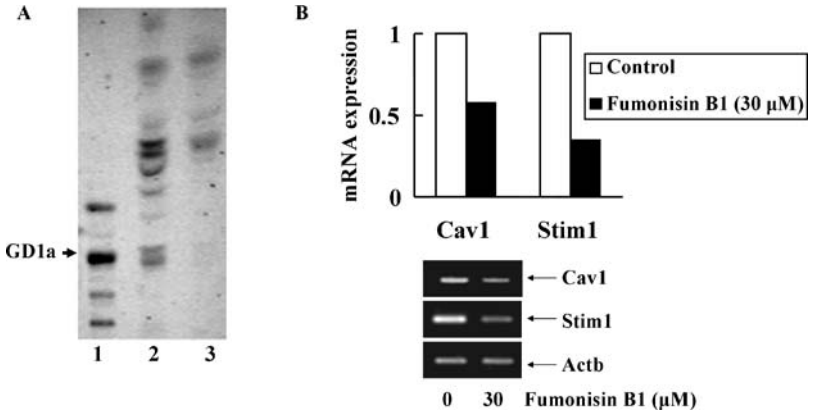

Fig. 7 Depletion of GD1a by fumonisin B1 suppresses expression of caveolin-1 and Stim1. FBJ-S1 cells were cultivated in the presence of fumonisin B1 for 15 days and RNA was extracted for RT-PCR. ***A***, HPTLC chromatogram. Lanes 1, Standard ganglioside mixture obtained from bovine brain; 2, FBJ-S1 cells cultured in the absence of fumonisin B1 as a control; 3, FBJ-S1 cells cultured in the presence of fumonisin B1. ***B***, Densitometric analysis of mRNA expression in FBJ-S1 cells. Ordinate represents mRNA in arbitrary units where the ratio of DNA of Cav1 or Stim1 to Actb of control cells incubated without fumonisin B1 is expressed as 1. Lower panels show PCR results.

To fully confirm in a different way the effects of GD1a on caveolin-1 and Stim1, RNA interference was used to bring about decrease in GD1a in cells. SiRNA was designed so as to target St3gal5 mRNA encoding CMP-NeuAc: lactosylceramide alpha-2,3-sialyltransferase (beta-galactoside alpha-2,3-sialyltransferase 5) responsible for GM3 synthesis from lactosylceramide. Two batches of transiently transfected cells were obtained with sialyltransferase expression suppressed by 20 and 50% in cells containing control siRNA. Caveolin-1 and Stim1 were noted to be suppressed by 35% and 55%, respectively, compared to cells transfected with control siRNA vector palsmid. This also clearly indicate GD1a to regulate the synthesis of caveolin-1 and Stim1: increase in GD1a leads to enhanced caveolin-1 and Stim1 synthesis, while decrease in this compound impairs caveolin-1 and Stim1 expression.

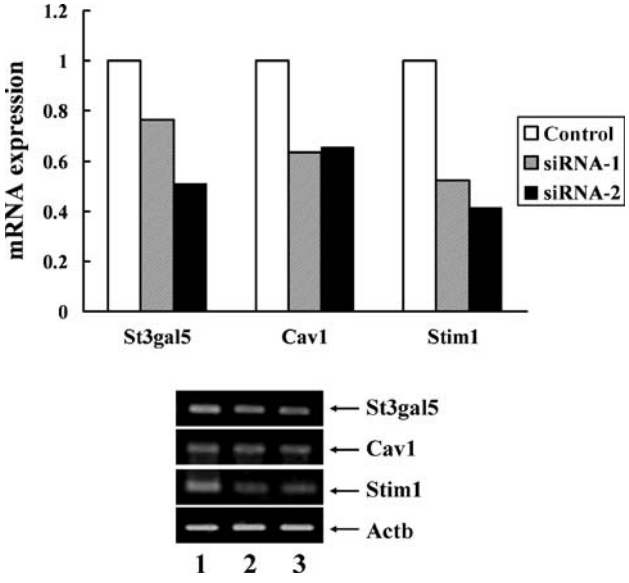

Fig. 8 Inhibition of caveolin-1 and Stim1 expression by siRNA against St3gal5. FBJ-S1 cells were transiently transfected with siRNA against St3gal5 (three target sequences) and control whose sequence is not present in mouse genome. At three days following transfection, RNA was extracted and assayed for expression of St3gal5, caveolin-1 and Stim1. Results of three out of four transfections are given. Lower panels show PCR results. Lane 1 represents control siRNA; lane 2, siRNA sequence 1; lane 3, siRNA sequence 2.

Discussion

To the best of the knowledge of the authors, this paper demonstrates for the first time ganglioside GD1a involvement in caveolin-1 and Stim1 synthesis. A reciprocal relationship between caveolin-1 expression and malignancy as tumor cells has been shown to exist [23,25,39]. Though both cell lines are malignant tumors, non-metastatic FBJ-S1 cells were found in this study to be rich in caveolin-1 compared to highly metastatic FBJ-LL cells. The transfectant expressing GD1a to the same extent as FBJ-S1 with no indication of metastasis subsequent to transplantation in mice [3] produces more caveolin-1 than metastatic mock cell, M5. These findings are consistent with the above relationship and further show caveolin-1 expression to be regulated by GD1a. In addition to enhanced expression of GD1a associated with elevated caveolin-1 synthesis, exogenous GD1a addition to FBJ-LL and M5 cells increases caveolin-1 mRNA and protein expression. But then, suppression of GD1a synthesis by fumonisin B1 and St3gal5 siRNA decreases the synthesis of caveolin-1.

To examine the effects of exogenous GD1a, cells were incubated in medium lacking serum, since the hydrophobic ceramide moiety of GD1a favors binding to hydrophobic serum albumin with consequent blockage of GD1a incorporation by cells. Serum deletion from the culture medium did not favor cell survival, possibly owing to the lack of growth factors. Incubation of cells with GD1a in the absence

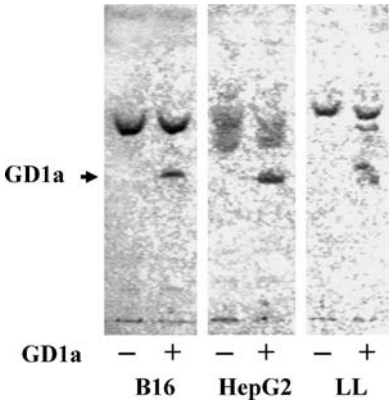

Fig. 9 HPTLC patterns showing incorporation of GD1a from the culture medium. B16 cells were incubated with 25 μM; HepG2 cells, 50 μM; and FBJ-LL cells, 100 μM GD1a in the absence of serum for 4 hours, supplemented with serum and further cultivated for 20 hours in serum-containing medium. This procedure was repeated once. Lipids were extracted from 3×10^6 cells and developed to separate gangliosides by HPTLC. Using standard GD1a developed simultaneously on the plate, it was calculated that B16 cells were found to incorporate 0.33 μg GD1a; HepG2 cells, 0.43 μg; FBJ-LL cells, 0.82 μg. These values are comparable to 0.76 μg GD1a of FBJ-S1 cells (3×10^6 cells).

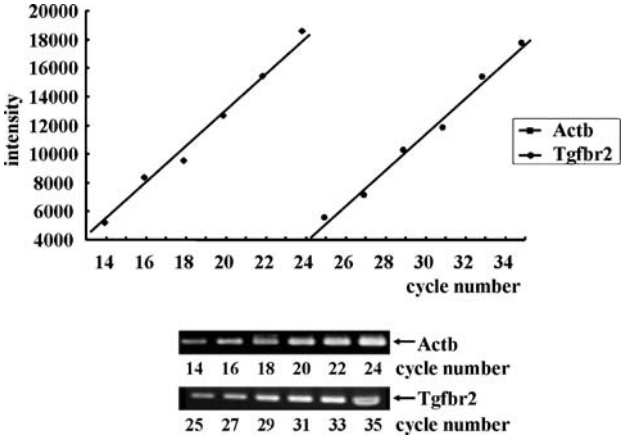

Fig. 10 Linearity of RT-PCR of Tgfbr2 and Actb (β-actin). RNA was extracted from FBJ-M5 and 1 μg RNA was subjected to RT-PCR using primers for Tgfbr2 and separately for Actb. After electrophoresis, gel staining band intensity assessment was made using an image analyzer.

of serum for 4 to 24 h provided uncertain results. Cells were thus treated with GD1a for 4 h in a serum-free RPMI-1640 medium, followed by incubation for 20 h in the presence of serum, resulting in greater synthesis of caveolin-1 and the effects of GD1a were confirmed for cells treated once more (two days) by this procedure. Caveolin-1 mRNA increased dose and time dependently. Whether 4 h GD1a treatment is the shortest possible time for signal stimulation of caveolin-1 synthesis remains to be confirmed. Pitto *et al*. found 4 h incubation sufficient to cause photolabeled GM1 association with caveolin-1 in A-431 cells [40]. Cells should at least incorporate GD1a and be kept healthy so as to express the effects of GD1a. At two days incubation, HPTLC indicated GD1a incorporation into cells to the same extent as FBJ-S1 cells which naturally express GD1a (Fig. 9). Cells containing GD1a thus show the effects of this compound.

GD1a depletion was carried out through use of inhibitors such as D-PDMP which suppresses the synthesis of glucosylceramide, fumonisin B1 which inhibits dihydroceramide synthesis and siRNA which inhibits sialyltransferase essential for GD1a synthesis. D-PDMP is widely used for glycosphingolipid synthesis suppression in attempt to clarify glycosphingolipid functions [41]. D-PDMP was found to decrease caveolin-1 by half, but cell health appeared compromised. Fumonisin B1 inhibits glycosphingolipid synthesis in a manner different from D-PDMP and has been used to clarify glycosphingolipid functions associated with axon formation [42]. Fumonisin B1 completely depleted ganglioside from FBJ-S1 cells, causing suppression of caveolin-1. St3gal5 siRNA was targeted to mRNA encoding sialyltransferase responsible for GM3 synthesis from lactosylceramide, inhibiting caveolin-1 mRNA expression. Diminished St3gal5 gene expression may possibly induce GM1b synthesis instead of GD1a. All these findings show ganglioside GD1a to regulate the synthesis of caveolin-1 in FBJ cells. Moreover, it is also to be said that all these findings are equally applicable to Stim1, a tumor suppressor protein like caveolin-1 [32,33] and recently shown essential for Ca^{2+}-store-depletion-mediated Ca^{2+} influx [34,35]. The same was noted for mouse melanoma B16 and human hepatocarcinoma HepG2 cells, the treatment of either of which with GD1a stimulated the synthesis of caveolin-1 and Stim1. The present study thus indicates GD1a to be the responsible factor for enhancing caveolin-1 and Stim1 expression in cells.

Caveolin-1 is a major constituent of caveolae in which cholesterol, sphingolipids and signal transducing molecules are present. Gangliosides are found primarily in caveolae and are closely associated with signaling molecules [43,44] and caveolin-1 [40,45]. But**,** glycosphingolipids may possibly be present in the raft, in contrast to caveolae. Sonnino *et al*. showed ganglioside-enriched domains to clearly differ from caveolae in MDCK II and human fibroblast cells [46]. Biochemical separation using mouse B16 cells containing GM3 as the sole glycosphingolipid indicated GM3 to have no association with caveolin-1 [47]. The detergent insoluble fraction was separated into one bound to anti-GM3 and another bound to anti-caveolin antibodies. The former contained GM3, sphingomyelin, cholesterol, cSrc and RhoA, and the latter, no GM3, cSrc or RhoA, but caveolin, large quantities of cholestrol and Ras. The binding of B16 cells to Gg3-coated plates by GM3-dependent adhesion enhanced the activation of cSrc and Fak. For ganglioside GD3 and Lyn with TAG-1, a glycosylphosphatidylinositol-anchored neuronal cell adhesion molecule [44], cell-surface glycosphingolipids degradation by endoglycoceramidase [48] induced a different TAG-1 distribution on a sucrose density gradient

and reduced TAG-1-mediated Lyn activation, suggesting possible glycosphingolipid involvement in signal trasmsduction in lipid rafts, a possibility supported by the overexpression of GM3 which induces caveolin-1 shift from the detergent-insoluble to detergent-soluble fraction [49]. GM3 overexpression caused GM3 clustering on the cell membranes of keratinocyte-derived cells and promoted coimmunoprecipitation of caveolin-1 and GM3 with the epidermal growth factor receptor.

The mechanism by which increase in GD1a on FBJ cells stimulates caveolin-1 and Stim1 synthesis remains to be clarified. Signaling pathways, such as p42/44 MAPK, nonreceptor tyrosin kinases and PKA, transcriptionally downregulate caveolin-1 gene expression [19]. A previous study showed GD1a to suppress the phosphorylation of c-Met [2] with possibly consequent diminished p42/44 MAPK activation. Treatment of FBJ-S1 cells with U0126 (MEK inhibitor) was found not to affect caveolin-1 mRNA synthesis[1] and thus c-Met phosphorylation inhibition by GD1a may not be involved in the synthesis enhancement of caveolin-1.

In the study of Ladisch *et al*., pretreatment with GD1a stimulated the proliferation of normal human dermal fibroblasts and Swiss 3T3 fibroblasts [50]. GD1a enrichment of cell membranes increased EGFR dimerization and GD1a enrichment triggered greater EGFR dimerization in the absence of the growth factor, resulting in enhanced activation of the EGFR signal transduction cascade subsequent to EGF addition [51]. GD1a in FBJ cells would not likely behave in the same manner as the above cells, since cell growth rates for FBJ-S1, rich in GD1a and FBJ-LL cells, all devoid of GD1a, are basically the same [1]. Too, activation of the growth factor receptor leading to the p42/44 MAPK activation may down-regulate caveolin-1 synthesis as shown above. EGFR dimerization induced by GD1a is thus not explanation for the enhanced synthesis of caveolin-1.

The expression of certain tumor related genes such as caveolin-1 and Stim1 was determined by RT-PCR. Ncam1, Itgb5, Tgfb1, Tgfbr2, Catna1, Rab7 and Twist1 mRNA expression in metastatic FBJ-LL and non-metastatic FBJ-S1 cells was virtually the same (Fig. 1) and there was no Icam1, Cdh1, Cdh6, Cdh3 or AW551984 expression in FBJ cells, indicating these tumor related genes not to be involved in the regulation of metastasis by GD1a or related to elevated synthesis of caveolin-1 and Stim1. Still, there is the possibility that molecules other than caveolin-1 and Stim1, with expression regulated by GD1a, have involvement in metastatic machinery of FBJ-LL cells. RhoC was found overexpressed in aggressive cancers where less caveolin-1 was present. Inhibition of RhoC or P38 MAPK brought about the partial restoration of caveolin-1 expression, which thus may possibly be partially regulated by some pathway involving RhoC [52].

Other gangliosides such as GM1, GD3 and GD1b, though not GT1b, had little effect on caveolin-1 and Stim1 synthesis, indicating ganglioside effects on this synthesis to be structurally specific. Gangliosides at low concentration activate calmodulin-dependent enzymes by binding to molecules at the calmodulin-binding site and at higher concentration, inhibit the enzyme by binding to calmodulin-like sites [53,54]. In interaction with phosphodiesterase, GT1b was shown to have stronger affinity toward the calmodulin-binding site of phosphodiesterase, followed by GD1a. The effects of GD1a may thus reasonably be considered mimicked by GT1b, provided GD1a binds to molecules in the transducing of signals in FBJ cell. Clarification of the mechanism by which GD1a regulates caveolin-1 and Stim1 synthesis should serve to elucidate this matter in greater detail.

Complete depletion of GD1a from cells failed to bring about the full suppression of caveolin-1 and Stim1 transcription, so that there are at least two pathways for the synthesis of caveolin-1 and Stim1: one controlled by GD1a and the other, unrelated to GD1a. That GT1b downregulated Stim1 synthesis in FBJ-M5 and Stim1 was not upregulated in FBJ-LL by GD1a treatment, suggests Stim1 synthesis to be regulated in a manner different from caveolin-1 synthesis by GD1a. Study is presently under way to determine signaling molecules involved in FBJ cells, with consequent enhancement of caveolin-1 and Stim1 synthesis.

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