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Structural characterization and dynamics of globotetraosylceramide in vascular endothelial cells under TNF-α stimulation

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Abstract In several vascular inflammatory reactions (*i.e.* immunity and thrombosis) inflammatory mediators lead to the activation of vascular endothelial cells (EC). To date, a number of functional molecules induced on the surface of activated-EC have been identified. We report here that Globotetraosylceramide (Gb4), a glycosphingolipid expressed in EC, is a novel inducible molecule on EC activated by TNF-α. The cell surface expression of Gb4 is increased in a time-dependent manner under TNF-α stimulation, which shows distinct expression kinetics of major proteins induced by TNF-α on EC. MALDI-TOF-MS analysis revealed that the enhanced Gb4 predominantly contains C24:0 fatty acid in the ceramide moiety. Isolated caveolae/lipid raft-enriched detergent insoluble membrane domains in activated-EC predominantly contain this molecular species of Gb4. Gb4 containing C16:0 fatty acid in the ceramide moiety, which is known to constitute the major species of Gb4 in plasma, is also found as a major molecular species in EC. These observations indicate that Gb4, especially with very long fatty acid, is enhanced in EC during its inflammatory reaction, and suggest the potential utility of Gb4 as a biomarker for monitoring inflammation status of EC involving its related diseases.

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Abbreviations

DIM	detergent insoluble membrane
EC	vascular endothelial cell
GA1	asialo-GM1 (Galβ1-3GalNAcβ1-4LacCer)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gb3	globotriaosylceramide (Galα1-4LacCer)
Gb4	globotetraosylceramide (GalNAcβ1-3Galα1, 4LacCer)
GM3	NeuAcα2-3LacCer
GSL	glycosphingolipid
HUVEC	human umbilical vein endothelial cell
IL-1β	interleukin-1β
LacCer	lactosylceramide (Galβ1-4Glcβ1Cermide)
LPS	lipopolysaccharide
RBC	red blood cell
TNF-α	tumor necrosis factor-α

Introduction

Glycosphingolipids (GSLs) are ubiquitously expressed in mammalian cells. To date, a wide variety of glycan structures have been identified in GSLs derived from mammalian tissues. Globo-series GSLs are characterized by the presence of an α1-4-galactose structure (Fig. 1). The initial structure of globotriaosylceramide (Gb3) is synthesized by α1,4-galactosyltransferase (Gb3 synthase) from its precursor lactosylceramide and UDP-galactose [1]. Gb3 has been characterized as CD77 antigen expressed on a subset of immature B-cells [2] or Burkitt's lymphoma [3] and has also been identified as blood group P^k antigen [4].

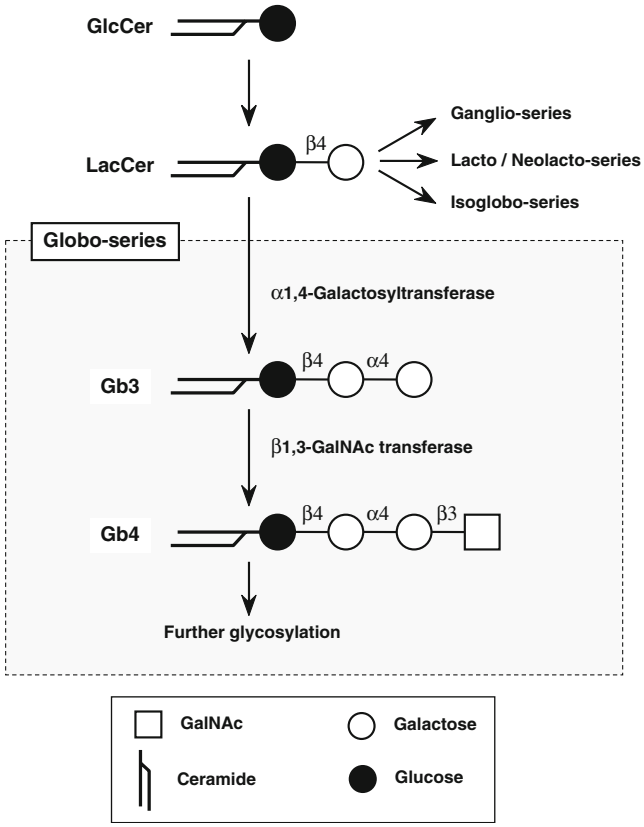

Fig. 1 Schematic representation of the synthetic pathway of globo-series GSLs in mammalian cells

Globotetraosylceramide (Gb4), also known as globoside, is synthesized by β1,3-N-acetylgalactosaminyltransferase (Gb4 synthase) from Gb3 and UDP-GalNAc [5]. Gb4 was originally characterized as the principal GSL in human erythrocytes [6] and has been identified as the blood group P antigen [4]. Gb4 is further glycosylated through a sequential series of enzyme reactions in cells and develop into other globo-series GSLs.

Vascular endothelium is one of the tissues that express globo-series GSLs [1, 7–11]. Moreover, up-regulation of Gb3 levels has been observed in primary-cultured vascular endothelial cells (EC) after stimulation by inflammatory mediators, such as LPS, IL-1β and TNF-α. This event is known to be involved in cytotoxicity of bacterial toxins that integrate into cells through binding to Gb3 on the cell surface [7, 8], and also suggests the novel aspect of globo-series GSLs (Gb3 including its derivatives in EC) as a inducible molecule in EC under inflammation. However, to date, there is a paucity of data concerning that alteration of globo-series GSLs in EC during inflammatory stimulation.

The characterization of GSLs in primary cultured EC using general detection methods, such as thin-layer chromatography followed by orcinol-H_2SO_4 or resorcinol-HCl staining, is technically difficult. Indeed, due to low growth rate of primary cultured cells, it is often problematic preparing sufficient quantities of GSLs for the general colorimetric detection methods. Previously, an approach for resolving this issue by using a unique mass culture system was reported [9, 10]. Although this technique was successful in determining the structural details of GSLs from a vast amount of primary-cultured endothelial cells, it is by no means a universal methodology. Hence, the information of the alteration of globo-series GSLs in EC under inflammation is limited to the increased expression of Gb3, which could be sensitively detected by the binding of its recognized toxins [7, 8].

To circumvent these problems, we have developed a highly sensitive generic analysis system for globo-series GSLs involving HPLC, MALDI-TOF-MS and flow cytometry. These analyses identified Gb4, especially with very long chain fatty acids, as a predominant GSL on the EC surface upon induction by TNF-α.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC), purchased from KURABO (Osaka, Japan), were maintained in the HuMedia-EG2 (KURABO). Passages 4-9, were used in these experiments. When stimulated by TNF-α (PeproTech, Rocky Hill, NJ), 5 x 10^5 cells were seeded on a culture dish (100 mm diameter) and then incubated for 24 h. After incubation, medium was replaced with fresh HuMedia-EG2 containing 20 ng/ml of TNF-α, and incubated for another 24 h. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Glycolipid extraction

Glycolipid extraction was performed as reported previously [12]. Briefly, total lipids from 1×10^7 cells were sequentially extracted with the chloroform/methanol/water 2:1:0 and 1:2:0.8 (*v*/*v*/*v*), respectively. Gangliosides and neutral glycolipids were separated by column chromatography using DEAE Sephadex A-25 (Sigma-Aldrich, St. Louis, MO) and Iatrobeads 6RS-8060 (Mitsubishi Kagaku Iatron, Tokyo, Japan), respectively.

HPLC analysis

Semi-quantitative analysis of GSLs was carried out by HPLC using a published carbohydrate fluorescent labeling method for GSLs [13] with slight modification [12]. Neutral GSLs or gangliosides from 1×10^6 cells or 10 μg

of GSL standards in chloroform/methanol (2:1, *v/v*) were evaporated to dryness in a glass vial. The carbohydrate moieties were then digested by incubation in the presence of 4 mU recombinant endoglycoceramidase II (Takara Bio) at 37°C for 16 h in 10 μl of 50 mM sodium acetate buffer (pH 5.0) containing 1 mg/ml sodium cholate. The liberated oligosaccharide was fluorescently labeled using anthranilic acid (2-AA, Sigma-Aldrich). Samples were sequentially mixed with 80 μl of labeling mixture (30 mg/ml 2-AA, 40 mg/ml sodium acetate trihydrate, 20 mg/ml boric acid, and 45 mg/ml sodium cyanoborohydride in methanol) and incubated at 80°C for 1 h. Labeled oligosaccharides were purified using a discovery DPA-6S column (Supelco, Poole, UK) and analyzed using a TSK gel-amide 80 column (Tosoh, Tokyo, Japan) and the HPLC system LC Module I plus (Waters, Milford, MA). The chromatography system and fluorescence detection/gradient conditions were identical to those described in a previous publication [13].

Immunofluorescence analysis

Cultured cells on poly-L-lysine coated glass plates were fixed with 3% paraformaldehyde in PBS for 5 min at RT and then permeabilized by 0.1% Triton X-100. The fixed cells were incubated with a rabbit anti-Gb4 polyclonal antibody (Matreya LLC, Pleasant Gap, PA) in PBS (1:100) and sequentially labeled with Alexa 488-conjugated anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA). Antibody binding was detected by immunofluorescence microscopy. Cell preparations treated only with secondary fluorescence antibody were used for monitoring the background.

Flow cytometry analysis

Expression of Gb4, E-selectin, VCAM-1 and ICAM-1 on the cell surface were analyzed by flow cytometry. After treatment with 20 ng/ml TNF-α, cells were harvested using 5 mM EDTA in PBS solution, and approximately 1×10^6 cells were suspended in 100 μl of cold PBS. For the detection of Gb4, the suspension was incubated with 1 μl of rabbit anti-Gb4 polyclonal antibody (Matreya LLC) on ice, and was sequentially labeled with Alexa 488-conjugated anti-rabbit IgG antibody (Invitrogen). For the detection of E-selectin, VCAM-1 and ICAM-1, the suspensions were incubated on ice with 20 μl of phycoerythrin labeled anti-human E-selectin mAb HCD62E (Biolegend, San Diego, CA) or anti-human VCAM-1 mAb STA (eBioscience, San Diego, CA) or anti-human ICAM-1 mAb HA58, respectively. The labeled cells were analyzed by a flow cytometer (FACS Calibur™, BD Biosciences, Franklin Lakes, NJ).

RT-PCR

RT-PCR analysis of glycosyltransferase genes was carried out according to the previously reported method [12]. Total RNA was isolated using Trizol reagent (Invitrogen) from HUVEC before and after treatment with 20 ng/ml of TNF-α. The amplification of the target gene cDNA was carried out by using gene specific primers and the SuperScript™ One-Step RT-PCR System with Platinum™ Taq DNA polymerase (Invitrogen) according to manufacturer's instructions. Briefly, total mRNA (0.5 μg) and forward and reverse primers (5 pmol each) were mixed with the SuperScript™ RT/Platinum *Taq* Mix (0.5 μl) in the reaction buffer (25.0 μl) containing dNTP (0.2 mM) and $MgSO_4$ (1.2 mM) in distilled water, and these were reacted in a thermal cycler. The reactions were performed using the following temperature cycling conditions: 55°C for 30 min, 94°C for 2 min, and then 40 cycle (for *Gb3S*) or 38 cycles (*Gb4S and B4GalT6*) or 32 cycles (for *GAPDH*) of 94°C for 15 s, 59°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 5 min. For amplification of *Gb3S* cDNA, the sense primer 5′-TGGAAGTTCGGCGGCATC TA-3′ and the antisense primer 5′-CAGGGGGCAGG GTGGTGACG-3′ were used. The PCR products corresponded to nucleotides +550 to +844 of the ORF region of the *Gb3S* gene. For amplification of *Gb4S*, *B4GalT6* and *GAPDH* cDNA, following primers were used; for *Gb4S,* the forward primer 5′-AGGTGTTCCCTCCATACTGCA-3′ and the reverse primer 5′-GTTCCTTAGCATGACCT GCCA-3′; for *B4GalT6,* the forward primer 5′-TGAACA GACTGGCACACAACC-3′ and the reverse primer 5′- TG TCAGCCCACTTACACCAC-3′; for *GAPDH*, the forward primer 5′-CCACCCATGGCAAATTCCATGGCA-3′ and the reverse primer 5′-TCTAGACGGCAGGTCAGGTCCA CC-3′. The PCR products were analyzed by agarose gel electrophoresis (1.5% gel) and the DNA was visualized using ethidium bromide under U.V. illumination. The intensities of bands of products in RT-PCR were quantified by scanning the bands using the Image Analyzer LAS-3000 (Fuji Film, Tokyo, Japan).

MALDI-TOF-MS

Mass spectrum analysis of GSLs was carried out by MALDI-TOF-MS according to a previously published method [14]. Purified Gb4 from human RBC (Sigma-Aldrich) and a commercially available Gb3 (Larodan Fine Chemicals, Malmö, Sweden) was used as a standard. A saturated solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) in a 1:1 mixture of acetonitrile/water containing 0.1% trifluoroacetic acid was used as the matrix solution. Neutral GSL purified from 1×10^7 cells (or DIM fraction isolated from 2×10^7 cells) or 1 μg of standard Gb4 were

dissolved in 2:1 (*v*/*v*) mixture of chloroform/methanol, and then co-crystallized in the α-CHCA solution. Samples were analyzed with Autoflex II (Bruker Daltonics, Leipzig, Germany) operated in the reflector mode. Peptide standards were used to achieve a six-point external calibration for mass assignment of ions.

Preparation of detergent insoluble membrane fraction

A detergent insoluble membrane (DIM) fraction was isolated from HUVEC stimulated by TNF-α using sucrose density gradient centrifugation in the presence of Triton X-100 [15]. In brief, cells were harvested and resuspended in Buffer A (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA) supplemented with 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride and a proteinase inhibitor cocktail (Complete mini EDTA-free™; Roche, Penzberg, Germany). The cells were homogenized sequentially using a tight fitting Dounce homogenizer (total of 15 strokes). The lysate was brought to a concentration of 40% (*w*/*v*) sucrose by the addition of 80% sucrose in Buffer A. After layering a linear sucrose gradient (5–30% sucrose in buffer A) over the lysate, the gradient was centrifuged for 16 h in a SW 32 Ti rotor (Beckman Coulter, Fullerton, CA) at 30,000 rpm, 4°C. Ten fractions were collected from top to bottom of the gradient. Protein markers for caveolae/lipid raft or actin protein in each fraction were detected by Western blotting using mouse IgG1 monoclonal antibodies (BD Biosciences), anti-caveolin 1 (mAb 2297), anti-flotillin 1 (mAb 18), anti-flotillin 2 (mAb 29) and anti-Actin (mAb C4) as appropriate.

Results

Changes of the expression level of GSLs in HUVEC under TNF-α stimulation

The expression levels of GSLs in HUVEC were analyzed by HPLC using a carbohydrate fluorescence labeling method [12, 13] as described in *Materials and Methods*. The carbohydrate portion in purified GSL was released by Endoglycosylceramidase II (rEGCase II, EC 3.2.1.123), and labeled with a fluorescence substance (anthranilic acid). Labeled-carbohydrates were separated by HPLC and the fluorescence intensities were quantified (Figs. 2 and 4). This methodology could clearly detect even small amounts of GSLs extracted from 1×10^5 cells. Our results indicate that lactosylceramide, Gb3 and Gb4 are major components of neutral GSL in the rested-HUVEC (Fig. 2, *middle panel*). Some unidentified minor components were also detected. From the elution time, these minor components seem to be Fucosyl-nLc4 and its derivatives, which are known to be expressed on HUVEC [11].

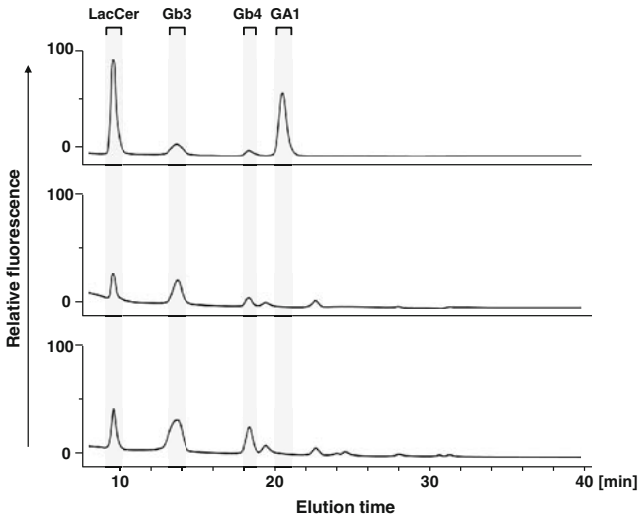

Fig. 2 HPLC analysis of neutral GSL-derived carbohydrates in HUVEC after TNF-α stimulation. Neutral GSLs purified from HUVEC (*middle panel*) or HUVEC stimulated by 20 ng/ml TNF-α for 24 h (*under panel*) were analyzed by HPLC using carbohydrate fluorescent labeling method as described in *Materials and Methods*. The elution pattern of standard 2-AA-labeled carbohydrates, which were generated from commercially available GSLs (LacCer, Gb3, Gb4, GA1) are shown in the *upper panel*. Samples equivalent to 1×10^5 cells of neutral GSLs or 25 ng of each standard were analyzed

After TNF-α stimulation for 24 h, the expression levels of neutral GSLs were markedly changed (Fig. 2, *bottom panel*). Notably, a strong enhancement of the Gb4 level was observed. These results are summarized in Fig. 3. Because globoseries-GSLs are strongly resistant to hydrolysis by rEGCase II [16], carbohydrates in Gb4 and Gb3 are partially released. Compared with other GSLs, 18.2% of Gb3 or 7.6% of Gb4 are hydrolyzed by our method (Fig. 2, *upper panel*). Thus we calculated the levels of Gb4 and Gb3 from the rates of hydrolysis. The corrected expression values are given in Fig. 3. Our results revealed that Gb4 is significantly up-regulated (3.0-fold induction) after TNF-α stimulation in EC. In addition Gb3, which is another abundant neutral GSL in rested-HUVEC, was up-regulated 1.9-fold after TNF-α stimulation. The level of other minor components, including lactosylceramide, displayed some slight increase in activated-EC. The majority of gangliosides in HUVEC are GM3 (Fig. 4). In addition, several minor components, which might be sialylparagloboside and their corresponding derivatives [11], were also detected. The expression level of each ganglioside remained unchanged after TNF-α stimulation.

Immunofluorescence analysis revealed that the majority of Gb4 is localized in the intracellular region of rested-HUVEC (Fig. 5). The fluorescence intensity was up-regulated after TNF-α stimulation, which was also associated with the enhancement of Gb4 localization in the plasma membrane (Fig. 5, *arrow heads*). No significant fluorescence was observed in the negative control (data not shown).

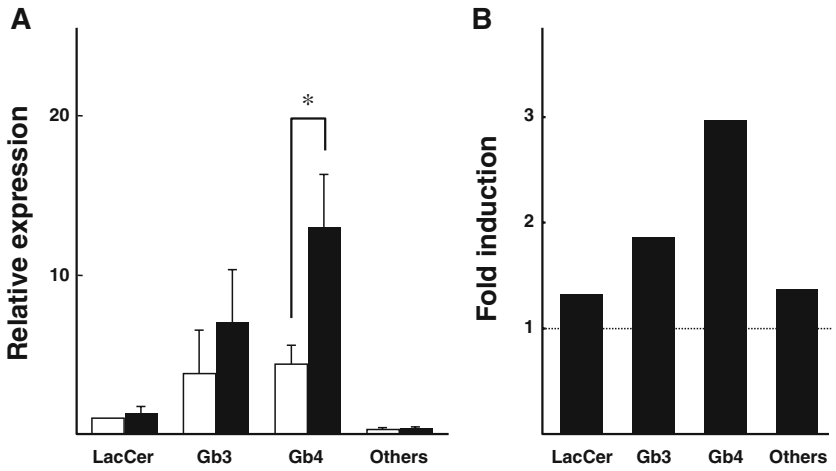

Fig. 3 Expression pattern of neutral GSL in HUVEC under TNF-α stimulation. **a** Relative expression levels of GSL in Figure 1 are represented as the ratio of LacCer expression levels in non-treated HUVEC. Open bars, non-treated HUVEC; closed bars, TNF-α-stimulated HUVEC. Error bars, mean ± SE, n=3, from three independent experiments. * P<0.05. **b** The increased levels of GSL in A are represented as fold induction (TNF-α-stimulated HUVEC / non-treated HUVEC)

Characterization of the expression kinetics of Gb4 on EC under TNF-α stimulation

To determine the expression kinetics of Gb4 on EC under TNF-α stimulation, we analyzed the time-dependent expressional change of Gb4 by flow cytometry (Fig. 6). We also examined the expression kinetics of major proteins induced by TNF-α [17–19]. E-selectin is a well known adhesion molecule which mediates leukocyte tethering on activated-EC by inflammatory mediators. ICAM-1 and VCAM-1, major adhesion molecules found on EC, are known to mediate strong adhesion between leukocytes and activated-EC. E-selectin and VCAM-1 showed almost identical kinetics on EC under TNF-α stimulation. These proteins are expressed on EC at an early stage of TNF-α stimulation, but are almost undetectable 12 h after the initial TNF-α treatment. ICAM-1 is also rapidly expressed on EC, although this protein is continuously expressed on EC under TNF-α stimulation. The level of Gb4 gradually increases after TNF-α stimulation, reaching a maximum after 12 h. The expression level of Gb3 on HUVEC is quite low even after TNF-α stimulation (data not shown).

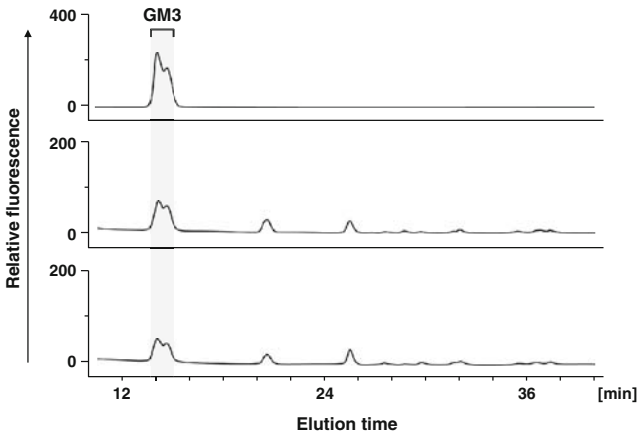

Fig. 4 HPLC analysis of acidic GSL-derived carbohydrates in HUVEC after TNF-α stimulation. Gangliosides purified from HUVEC (*middle panel*) or HUVEC stimulated by 20 ng/ml TNF-α for 24 h (*under panel*) were analyzed by HPLC using carbohydrate fluorescent labeling method as described in *Materials and Methods*. The elution pattern of standard 2-AA-labeled carbohydrates, generated from commercially available GM3 is shown in the *upper panel*. Samples equivalent to 1×10^5 cells of gangliosides or 100 ng of standard GM3 were analyzed

It has been reported that increased expression of Gb3 in EC by TNF-α stimulation is due to the transcriptional up-regulation of the Gb3 synthase gene (*Gb3S*) [20]. RT-PCR analysis (Fig. 7) showed that the *Gb3S* mRNA was markedly increased upon TNF-α stimulation under our experimental conditions. The other glycosyltransferase genes involved in Gb4 synthesis, the β1,4-galactosyltransferase gene 6 (β4GalT) which codes for a lactosylceramide synthase [21] and the Gb4 synthase gene (*Gb4S*) [5], were also up-regulated by TNF-α, although the rates were relatively low. These results suggest that the up-regulation of *Gb3S* mRNA is primarily responsible for the increased expression of Gb4 in EC upon stimulation by TNF-α.

Mass spectrum profiles of Gb4 in EC under TNF-α stimulation

The ceramide structure in Gb4 was determined by MALDI-TOF-MS analysis. Gb4 from human red blood cells (RBC) were used as a standard. In the positive-ion mass spectrum, molecular ions of $[M + Na]^+$ (m/z 1221.8 to 1361.9) corresponding to Gb4 were detected in neutral GSLs from HUVEC (Fig. 8, summarized in Table 1). Major peaks, 1249.8, 1359.9 and 1361.9, corresponding to Gb4 possess-

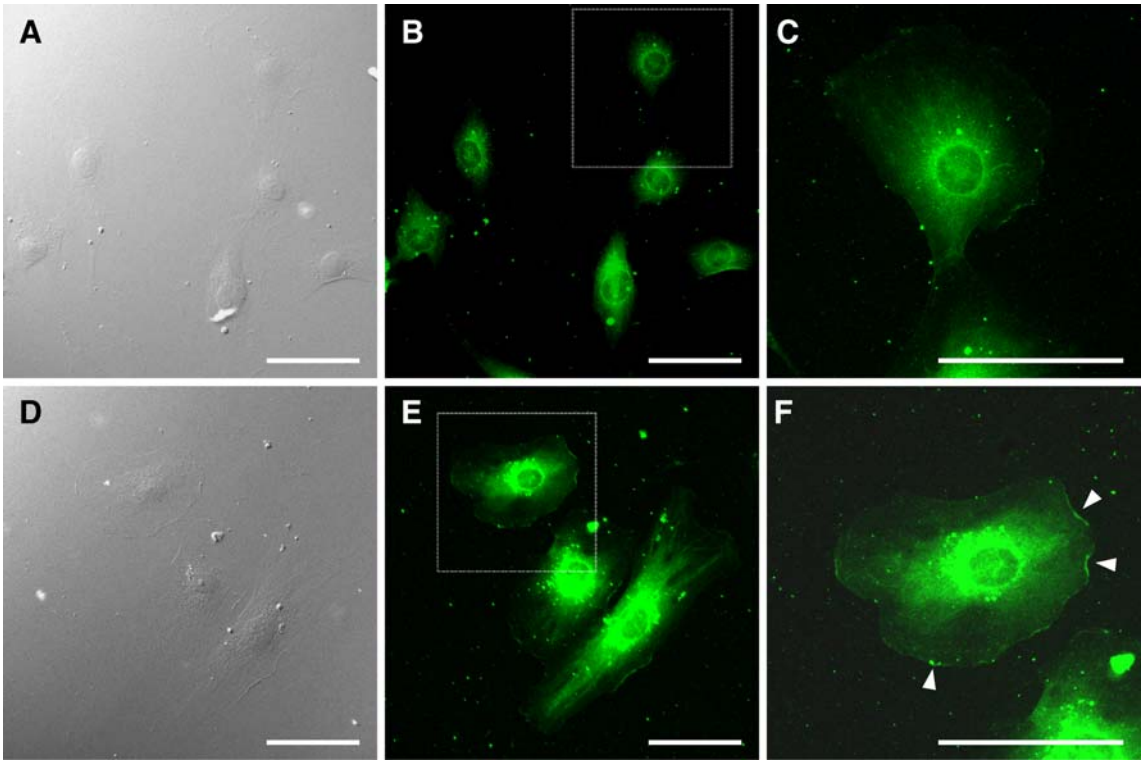

Fig. 5 Immunofluorescent analysis of Gb4 in HUVEC. Cells were stained with anti-Gb4 rabbit polyclonal antibody using Alexa 488-conjugated secondary antibody for the detection. Panels **a**–**c** show non-treated HUVEC. Panels **d**–**f** show HUVEC treated with TNF-α (20 ng/ ml) for 24 h. Panels **a** and **d** show Nomarski images of **b** and **e**, respectively. Panels **c** and **f** are high magnification images of marked areas in panels **b** and **e** (*open squares*), respectively. Arrowheads indicate Gb4 expression on the edge of HUVEC (plasma membrane). The scale bars indicate 50 μm

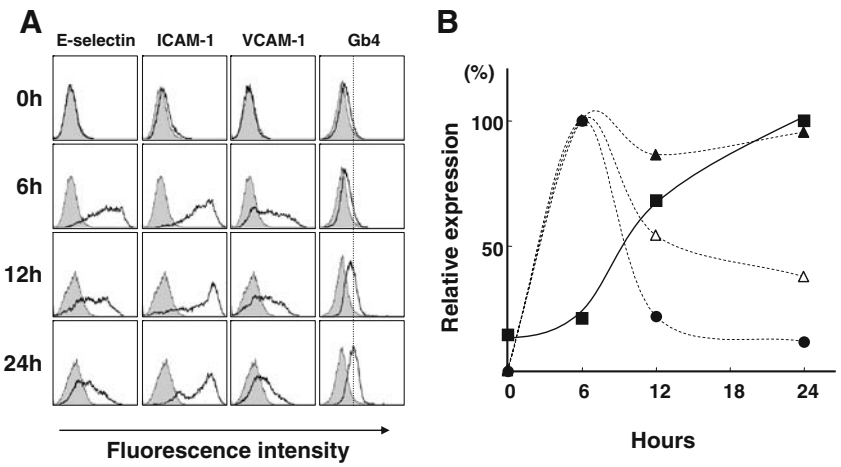

Fig. 6 Flow cytometry analysis of Gb4 and multiple adhesion molecules on the surface of HUVEC under TNF-α stimulation. **a** Flow cytometric patterns of E-selectin, ICAM-1, VCAM-1 and Gb4 on HUVEC. Cells were stimulated by incubation with 20 ng/ml TNF-α for a specified period of time and then stained with the relevant antibody as described in *Materials and Methods*. Cells stained with each antibody are displayed by *thick lines*. Controls were prepared with the second antibody alone and displayed by *thin lines with dark shading*. **b** Expression levels of E-selectin, ICAM-1, VCAM-1 and Gb4 on HUVEC are shown on the basis of geometric means of fluorescence intensities. The geometric mean fluorescence values were subtracted from those of the corresponding controls. Values are given as the percentage of the maximum level of fluorescence signal. E-selectin, *closed circle*; ICAM-1; c*losed triangle*, VCAM-1; *open triangle*; Gb4, c*losed square*

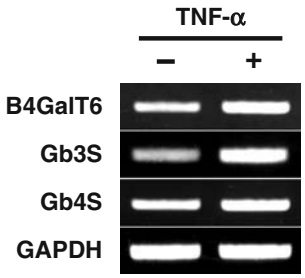

Fig. 7 RT-PCR analysis of the glycosyltransferase genes involving Gb4 synthesis in HUVEC under TNF-α stimulation. The expression levels of mRNAs cording for the glycosyltransferase genes involving Gb4 synthesis were analyzed by RT-PCR. Total mRNAs purified from HUVEC treated with (+) or without (−) TNF-α (20 ng/ml) for 12 h were used in this experiment. Expression of *GAPDH* mRNA in the cells was monitored as an internal control. *B4GalT6*, β1,4-galactosyltransferase 6 gene; *Gb3S*, Gb3 synthase gene; *Gb4S*, Gb4 synthase gene

Table 1 Gb4 from RBC and HUVEC characterized by MALDI-TOF MS

$[M + Na]^+$	Ceramde	Relative rate (%)		
		RBC	HUVEC	
			Non-treated	TNF-α-treated
1221.8	C14:0	n.d.	9.91	7.26
1249.8	C16:0	1.65	37.58	22.56
1331.8	C22:1	3.44	n.d.	n.d.
1333.8	C22:0	17.48	6.06	9.95
1359.9	C24:1	28.95	24.97	22.97
1361.9	C24:0	43.11	21.49	37.27
1387.9	C26:1	5.36	n.d.	n.d.

Relative rate indicates each of ceramide subspecies of Gb4 / total Gb4 × 100 (%). Values represent the average from three independent experiments. n.d.; not detected

ing 4-sphingosine with C16:0 fatty acid (C16:0-ceramide) or C24:1 fatty acid (C24:1-ceramide) or C24:0 fatty acid (C24:0-ceramide), respectively, were detected. The relative intensity of the peaks changed after TNF-α stimulation. Before stimulation, the predominant form of ceramide in Gb4 was C16:0-ceramide in HUVEC, but after stimulation, C24:0-ceramide became the predominant form. In addition, Gb4 possessing C16:0-ceramide is barely detectable in RBC by mass spectrometry analysis, although this structure is the major form of Gb4 in plasma [22]. Our results indicate that plasma Gb4 is primarily derived from EC rather than RBC.

The spectrum of molecular ions of $[M + Na]^+$ (m/z 1046.7 to 1158.8) corresponding to Gb3 were relatively weak, but nevertheless detectable, in neutral GSLs from HUVEC (Fig. 9; summarized in Table 2). The major molecular species of the ceramide in Gb3 were identical to the ceramide portion of Gb4, and also similarly altered after TNF-α stimulation in EC.

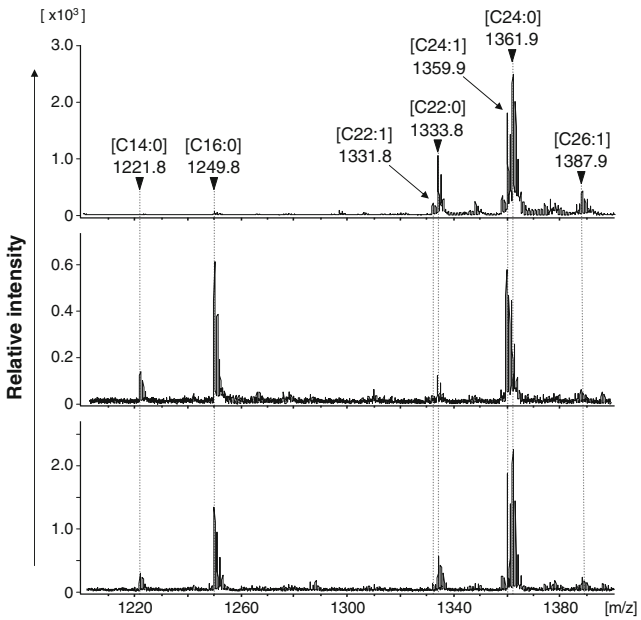

Fig. 8 Representative mass spectrum profiles of Gb4 from HUVEC. MALDI-TOF-MS analysis of neural GSLs from HUVEC (*middle panel*) or HUVEC stimulated by TNF-α for 24 h (*under panel*) was carried out as described in *Materials and Methods*. The mass spectrum profiles of standard Gb4 purified from human red blood cells (RBC) is shown in the *upper panel*

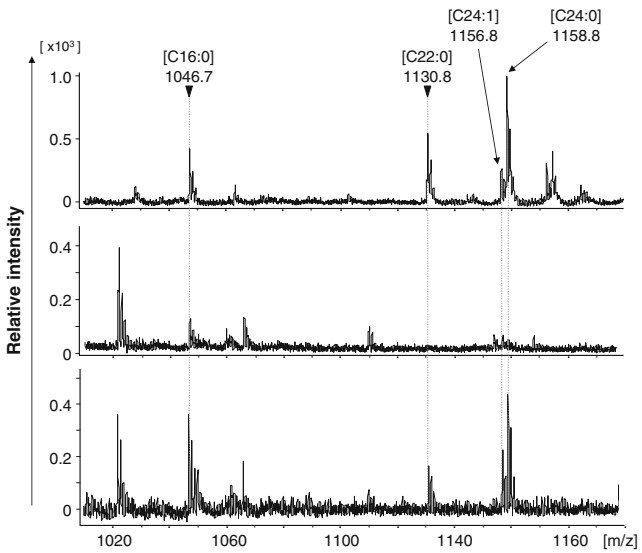

Fig. 9 Representative mass spectrum profiles of Gb3 from HUVEC. MALDI-TOF-MS analysis of neutral GSLs from HUVEC (*middle panel*) or HUVEC stimulated by TNF-α for 24 h (*under panel*). The procedure was carried out as described in *Materials and Methods*. The mass spectrum profiles of a commercially available standard Gb3 is shown in the *upper panel*

Table 2 Gb3 from HUVEC characterized by MALDI-TOF MS

$[M + Na]^+$	Ceramde	Relative rate (%)	
		Non-treated	TNF-α-treated
1046.7	C16:0	45.07	31.36
1130.8	C22:0	n.d.	14.50
1156.8	C24:1	28.24	20.91
1158.8	C24:0	26.69	33.22

Relative rate indicates each of ceramide subspecies of Gb3 / total Gb3 × 100 (%). Values represent the average from three independent experiments. n.d.; not detected

Next, we analyzed the MS spectra of Gb4 in the caveolae/lipid raft-enriched detergent insoluble membrane domains (DIM) isolated from activated-EC. The DIM were prepared by a sucrose density gradient analysis method using 1% triton X-100 described as *Materials and Methods*. By using several protein markers for caveolae (caveolin-1) and lipid rafts (flotillin-1 and flotillin-2), we determined the caveolae and lipid rafts were enriched between the 5% and 30% sucrose fraction (Fig. 10a, No. 2–3). We purified the Neutral GSLs from this fraction and analyzed them by MALDI-TOF-MS (Fig. 10b). The mass spectra of molecular ions of Gb4 $[M + Na]^+$ are clearly detected in the DIM fraction. The ratio of Gb4 possessing C24 fatty acid, especially C24:0 fatty acid in DIM (43%), is higher than that in the whole cell preparation (37%). The ratio of other molecular species of Gb4 in DIM is comparatively less than or equal to that of the whole cell preparation.

Discussion

Here we identified Gb4 as a major glycolipid induced by TNF-α on the cell surface of EC. Under TNF-α stimulation, expression levels of Gb4 are increased in a time-dependent manner, which is associated with a change of the main fatty acid in the ceramide moiety of Gb4 from C16:0 to C24:0. Namely, the level of Gb4 possessing C24:0 fatty acid is markedly increased in TNF-α activated EC under inflammatory conditions. Our results also confirmed that Gb3 is another major GSL in EC to be up-regulated upon TNF-α stimulation [7, 8], although its expression level on the EC surface is relatively low. These results indicate that Gb3 localizes intracellularly as a precursor of Gb4, and matures into Gb4 prior to transfer to the EC surface. Under TNF-α stimulation, transcription of *Gb3S* in EC is strongly promoted compared to other glycosyltransferase genes that are involved in Gb4 synthesis (Fig. 7). Moreover, the compositional changes to the fatty acid in Gb4 are almost identical to that in Gb3 (Tables 1 and 2). Taken together, our results suggest that Gb3 synthesis is accelerated in EC by TNF-α stimulation and that Gb3 matures into Gb4 before being recruited to the cell surface.

The important role of vascular endothelial cells in inflammatory reactions, immunity and thrombosis has been examined in recent years. In particular, progress has been made in understanding the regulatory mechanisms of leukocyte-EC interaction through the multiple adhesion molecules such as E-selectin [17], ICAM-1 [18] and VCAM-1 [19]. Inflammatory mediators, such as TNF-α, activate EC and induce expression of multiple adhesion molecules on the cell surface with variable kinetics. These molecules are sequentially expressed on activated-EC, which regulates the recruitment of leukocytes to inflammatory sites by rolling, firm adhesion and then transmigration [23].

Our results indicate that TNF-α activation of EC also increases the expression level of Gb4 on the cell surface during the late-stage of stimulation. To date, the role of Gb4 in leukocyte-EC interaction has not been reported. Our results raise the possibility that Gb4 regulates a stage-specific event of leukocyte-EC attachment during the inflammatory response. Further investigations will be conducted to reveal the detailed mechanism of this interaction.

It is known that GSLs make a complex with cholesterol and form a specialized area in the plasma membrane, which

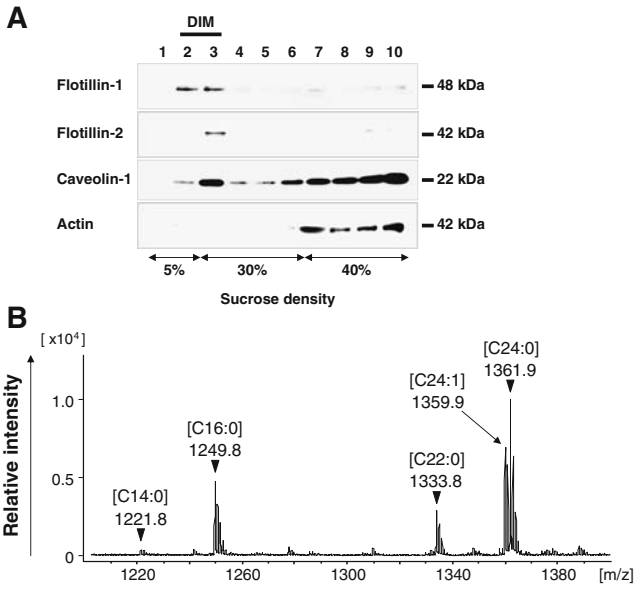

Fig. 10 Structural analysis of Gb4 in detergent insoluble membrane (DIM) of HUVEC stimulated by TNF-α. A DIM fraction was isolated by sucrose density gradient centrifugation from HUVEC stimulated by TNF-α as described in *Materials and Methods*. **a** Western blotting of several protein markers for the caveolae/lipid raft. Expression of actin protein was monitored as a control for the detergent soluble fraction. **b** Mass spectrum profiles of Gb4 in the DIM. Neutral GSLs purified from the DIM fraction were analyzed by MALDI-TOF-MS. Mass spectra of molecular ions of Gb4 $[M + Na]^+$ are shown

can be isolated as a detergent insoluble membrane (Fig. 10a). This complex, termed caveolae or lipid rafts, containing a number of functional molecules, such as tyrosine kinase, G protein and adhesion molecules, efficiently mediates outside-in signals at the plasma membrane [24]. Signaling events through the lipid rafts regulate leukocyte-EC interactions. For instance, ligation of E-selectin by its ligands expressed on leukocytes induces phosphorylation of phospholipase Cγ *via* the lipid rafts on EC, which in turn induces a downstream signaling cascade [25]. Because Gb4 is a major GSL in EC, any TNF-α induced up-regulation or structural changes associated with this molecule on the cell surface should influence signaling events mediated by the lipid raft. Our study revealed that the level of very long chain fatty acid (C24:0) containing Gb4 is markedly increased in EC under inflammation, and also demonstrated its localization in the lipid raft of activated-EC (Fig. 10). Such very long chain fatty acids containing GSL are known to be associated with acylated signaling proteins located at the inside of the plasma membrane *via* these fatty acids, and organize the lipid rafts for outside-in signal transduction [26]. The enhancement of Gb4 possessing very long chain fatty acid by TNF-α might contribute to the organization of the lipid rafts in EC to regulate signaling events for inflammatory responses.

Immunofluorescent analysis (Fig. 5) indicates that Gb4 localization in plasma membrane is associated with the increased expression of C24:0 containing Gb4. These results raise a possibility that the Gb4 localization in plasma membrane depends on the ceramide structure. Determination and analysis of the key gene for the synthesis of C24:0 containing Gb4 under TNF-α stimulation will be needed to reveal these hypothesis.

The results of MALDI-TOF-MS analysis also demonstrated that C16 fatty acid-containing Gb4 is a major GSL in EC. This structure has been observed in the majority of Gb4 in human plasma [22]. Because GSLs are known to be released from EC to plasma, our results indicate that Gb4 in human plasma is mainly produced by EC. By contrast, Gb4 from human RBC primarily contain C24 fatty acid. Indeed, Gb4 containing a C16 fatty acid species is barely detectable in human RBC (Fig. 8). Although RBC is known to be a source of plasma GSLs, this does not appear to be the case for the production of plasma Gb4 in human. These observations also suggest that the inflammatory status of EC is reflected in the fatty acid structure of plasma Gb4. Indeed, Gb4-mediated inflammatory signals might be a clinically useful diagnostic marker for the inflammatory status of EC. Recently, it has been reported that inflammation status of EC relates to cardiovascular diseases such as atherosclerosis [27].

In conclusion, our findings provide valuable new information concerning the inflammatory response in EC. We believe that progress in this field will contribute to a more complete understanding of the inflammatory response in EC and assist in the development of novel treatments for related human diseases.

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