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Globalotriaosylceramide induces oxidative stress and up-regulates cell adhesion molecule expression in Fabry disease endothelial cells

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Abstract

Fabry disease, an X-linked systemic vasculopathy, is caused by a deficiency of α -galactosidase A resulting in globalotriaosylceramide (Gb₃) storage in cells. The pathogenic role of Gb₃ in the disease is not known. Based on previous work, we tested the hypothesis that accumulation of Gb₃ in the vascular endothelium of Fabry disease is associated with increased production of reactive oxygen species (ROS) and increased expression of cell adhesion molecules. Gb₃ loading resulted in increased intracellular ROS production in cultured vascular endothelial cells in a dose-dependent manner. Increased Gb₃ also induced expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin. Reduction of endogenous Gb₃ by treatment of the cells with an inhibitor of glycosphingolipid synthase or α -galactosidase A led to decreased expression of adhesion molecules. Plasma from Fabry patients significantly increased ROS generation in endothelial cells when compared with plasma from non-Fabry controls. This effect was not influenced by reduction of intracellular Gb₃. This study provided direct evidence that excess intracellular Gb₃ induces oxidative stress and up-regulates the expression of cellular adhesion molecules in vascular endothelial cells. In addition, other factors in patient's plasma may also contribute to oxidative stress in Fabry vascular endothelial cells.

Keywords

Fabry disease; alpha-galactosidase A deficiency; globalotriaosylceramide; endothelial cells; reactive oxygen species; cell adhesion molecule

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Introduction

Fabry disease is an inborn error of glycosphingolipid catabolism caused by an insufficient activity of α -galactosidase A and progressive accumulation of globotriaosylceramide (Gb₃) and related glycosphingolipids (GSL) in the vasculature, including the vascular endothelium [1;2]. It is associated with a markedly increased risk of ischemic stroke of [3], manifold cardiac abnormalities and progressive renal failure.[4] The vascular complications are responsible for most symptoms and the premature mortality in Fabry disease. At present the pathophysiology of the disease is largely unknown.

Pathologically, vascular endothelial cells are the most predominantly affected cell type in this disease [2]. Clinical and animal studies demonstrated enhanced vascular thrombosis [5;6;7], and altered endothelial-dependent vascular reactivity in patients and in Fabry mouse model [8;9;10]. Increased markers of endothelial activation and endothelial microparticles were found in Fabry patients' plasma [11;12]. Others and we have found evidence for increased production of ROS [10;13;14].

We therefore hypothesized that Gb₃ accumulation leads to increased production of ROS and other pathological changes in cultured Fabry vascular endothelial cells.

Materials and Methods

Cell cultures and treatments

Microvascular endothelial cells were isolated and purified from a forearm skin biopsy from Fabry patients and non-Fabry controls as described previously [15]. These primary cells, primary dermal microvascular endothelial cells (HMVEC, Clonetics, Cambrex, Walkersville, MD) and a Fabry disease endothelial cell line with an extended life span, IMFE1 cells were maintained in EGM-2 medium (Clonetics) as described [15]. HMVEC and other primary endothelial cells at passage 3–5 and IMFE1 cells at passage 17 to 26 were used in this study. For EtDO-P4 treatment, EtDO-P4, was dissolved in dimethylsulphoxide (DMSO) (Sigma, St. Louis, MO), aliquoted and stored at -20°C until use. IMFE1 cells were maintained in EGM-2 medium containing $1\mu\text{M}$ EtDO-P4 for 10–21 days. At a concentration of $1\mu\text{mol/L}$, no significant changes in growth rate and morphology were observed. However, in a pilot study, a concentration of $2.5\mu\text{mol/L}$ led to significant IMFE1 cell death. For enzyme treatment, cells were incubated with recombinant α -galactosidase A (Shire Human Genetic Therapies, Cambridge, MA) at 0.08 IU/ml (1:500 dilution of original solution) for 4 days. Medium and enzyme were replaced daily. For tumor necrosis factor- α (TNF- α) treatment, cells were incubated with TNF- α (R & D systems, Minneapolis, MN) at 100 units/ml for indicated lengths of time (4 or 6 hrs).

Gb₃-loading

Gb₃-loading was performed essentially as described with modifications [16]. An Aliquot of Gb₃ (Matreya, Pleasant Gap, PA) dissolved in chloroform: methanol (2:1, v/v) was dried and DMSO was added. The mixture was heated at 90°C for 10 min with occasional vortexing. An appropriate amount of fatty acid-free bovine serum albumin (BSA, Sigma) in phosphate buffered saline (PBS) was added to obtain a 1:1 molar ratios of Gb₃:albumin. The Gb₃/albumin complex was sonicated for 5 min and was added to the medium containing 20% fetal bovine serum (FBS), 10 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, CA) and 100 $\mu\text{g/ml}$ sodium heparin (Invitrogen) in human endothelial serum-free medium (Invitrogen). Cultures of endothelial cells at approximately 80% confluence were incubated with the medium containing Gb₃/albumin complex for 2–3 days.

Human plasma-loading study

Blood samples were obtained from Fabry patients who were receiving enzyme replacement therapy (ERT) by intravenous infusions of α -Gal A (Shire Human Genetic Therapies, Cambridge, MA) every 2 weeks in Institutional Review Board of the National Institute of Neurological Disorders and Stroke-approved studies. Blood samples were taken immediately before enzyme infusion, (i.e. 14 days after the previous infusion) to minimize the effects of ERT on plasma. Plasma was aliquoted and stored at -80°C until use. Plasma was also obtained from healthy male volunteers and was used as controls. Confluent or near confluent endothelial cells were incubated for 2 days with 20% human plasma in human endothelial serum-free medium (Invitrogen) supplemented with 10 ng/ml bFGF (Invitrogen) and 100 $\mu\text{g/ml}$ sodium heparin (Invitrogen).

Immunostaining

Fluorescence immunostaining against Gb₃ was performed as described previously with modification [15]. After fixation with 2% paraformaldehyde and permeabilization with 0.3% Triton X-100, PBS was substituted for 0.01% Triton X-100 in all subsequent steps. We found that this omission of Triton X-100 moderately increases sensitivity of detection and that almost IMFE1 cells are positive for Gb₃-immunoreactive signals using this procedure.

Thin-Layer Chromatography (TLC)

Glycosphingolipid extraction was performed as described previously [17]. Protein concentration was determined by BCA protein assay reagent (Pierce, Rockford, IL). Lipid extract corresponding to equal amounts of cell proteins were loaded on TLC plates (Si-HPF, Baker, Phillipsburg, NJ) and were separated for 45 min in a solvent system consisting of chloroform/methanol/water (65/25/4). GSL were detected by 0.1% 5-hydroxy-1-tetralone (Sigma) prepared in 80% sulfuric acid as described [18] and fluorescence was visualized and quantified using the Storm imaging system (Amersham Bioscience, Piscataway, NJ).

Determination of intracellular ROS: H₂DCFDA dye assay

Intracellular ROS was monitored using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Molecular Probes, Eugene, OR) a cell-permeable indicator for ROS. H₂DCFDA is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Confluent cells grown on 96 well plates were washed and were incubated with 10 μM H₂DCFDA in Hank's balanced salt solution (HBSS) supplemented with 0.14 mg/ml CaCl₂, 0.1 mg/ml MgCl₂, 0.1 mg/ml MgSO₄, 2% FBS, 10 ng/ml bFGF and 2mmol/L L-arginine for 40 min at 37°C in the dark. The dye was removed and 100 $\mu\text{l/well}$ of the HBSS buffer was added to the cells and fluorescence was measured in a Victor plate reader (Perkinelmer, Waltham, MA) using excitation wavelength of 485 nm and emission wavelength of 535 nm. The fluorescence was measured every 2.5 min for up to 10 min and the rate of DCF signal increment was calculated and was used to express intracellular ROS level. ROS level was normalized for cell number determined by MTT assay or cell protein level. MTT assay was performed as described previously [15]. Protein concentration was determined using Micro BCA protein assay reagent (Pierce). Briefly, cells were rinsed with PBS, and were lysed by the addition of 25 $\mu\text{l/well}$ 0.05 N NaOH. After addition of BCA reagents (150 $\mu\text{l/well}$), the plate was incubated at 37°C for 40 min and absorbance was measured at a wavelength of 562 nm.

Quantitative RT-PCR

Quantitative real time RT-PCR was performed as previously described [15]. Pre-designed TaqMan probes and primers for human ICAM-1, VCAM-1 and E-selectin were purchased

from Applied Biosystems (ABI, Foster City, CA). 18S rRNA was used as internal control and detected by TaqMan probe and primers (ABI).

Western blot analysis

Western blot was performed as previously described [15] except for that denaturation and separation of the samples were done under non-reducing conditions, and that 10% Bis-Tris NuPAGE gels (Invitrogen) were used to separate proteins. Primary antibodies used were: mouse monoclonal antibody to human ICAM-1 (R & D systems) and rabbit polyclonal antibody to human VCAM-1 (H-276; Santa Cruz Biotechnology, Santa Cruz, CA). Actin was detected with a monoclonal antibody to β -actin (Sigma) as loading control.

Results

Gb₃ increases ROS generation in cultured Fabry endothelial cells

To study whether Gb₃ accumulation can lead to the excessive generation of ROS, we initially assessed ROS production in primary cultured endothelial cells from Fabry patients and control subjects. Enzyme assay showed that Fabry endothelial cells ($n = 3$) had significantly reduced α -galactosidase A activities compared to non-Fabry controls ($n = 3$) (0, 0 and 6.6 nmol/mg/hr in Fabry cells vs. 56.7, 74.6 and 132.55 nmol/mg/hr in controls). In immunostaining for Gb₃, Fabry endothelial cells showed characteristic punctate signals demonstrating lysosomal accumulation of Gb₃ and no significant positive signal seen in control endothelial cells (Fig. 1A). No significant difference in ROS was shown between the two groups (Fig. 1B). We considered that this was due to the significant heterogeneity in genetic background of donors, the rapid senescence of primary vascular endothelial cells in culture, and the limited number of available primary lines.

We therefore decided to study the biological effects of different amounts of Gb₃ in a well-characterized Fabry vascular endothelial line with extended lifespan, IMFE1 [15]. Three days after loading, cell-associated Gb₃ in lipids extracts increased significantly when analyzed by TLC (Fig. 2A). Gb₃ immunostaining showed higher intensities of positive intracellular signals in loaded cells as compared to untreated cells (Fig. 2B). This suggested that Gb₃ added to the medium was taken up by the cells then transported and accumulated in endosomes and/or lysosomes. Increased intracellular Gb₃ led to significantly increased ROS generation in the cells in a dose-dependent fashion (Fig. 2C). Treatment of IMFE1 with antioxidant, vitamin C (100 μ mol/L) significantly decreased intracellular ROS level. No significant difference in ROS production could be detected between cells with and without Gb₃-loading in the presence of vitamin C in the culture media (Fig. 2C).

Gb₃ up-regulates the expression of adhesion molecules

IMFE1 cells were incubated with 10 μ mol/L Gb₃ for 2 days and mRNA levels of ICAM-1, VCAM-1 and E-selectin were measured by quantitative RT-PCR. Gb₃-loading led to significant increment (up to 55% increase) of steady state mRNA levels of all three molecules (Fig. 3). Besides steady state, mRNA levels of these CAMs were also determined after TNF- α stimulation. TNF- α increased CAMs expression markedly. Gb₃ significantly enhanced TNF- α -induced ICAM-1 expression when compared with unloaded cells (Fig. 3).

Treatment of IMFE1 cells with 1 μ mol/L of the GSL synthase inhibitor EtDO-P4 [19] resulted in significant decrement of Gb₃ (Fig. 4A and B). EtDO-P4 also decreased Monosialoganglioside GM3 (GM3) but had minor effect on lactosylceramide (LacCer) and globosides (Gb₄) (Fig. 4A and B). This heterogenic effect may be due to different turnover and uptake rates from the medium of individual GSL. Consistent to the observations on TLC, there was significant reduction of Gb₃-immunoreactive signals in EtDO-P4-treated cells in

immunostaining (data not shown). In EtDO-P4-treated cells, VCAM-1 mRNA decreased significantly compared with untreated cells in both steady state and after TNF- α -stimulation (Fig. 4C), while the steady state mRNA level of ICAM-1 was increased in EtDO-P4-treated cells.

Treatment of IMFE1 cells with 0.08 IU/ml α -galactosidase A for 4 days significantly reduced Gb₃ as shown by immunostaining (Fig. 4D). After enzyme treatment, steady state ICAM-1 mRNA level decreased moderately, but to a significant extent ($P < 0.05$). However, α -galactosidase A treatment enhanced TNF- α -induced ICAM-1 expression (Fig. 4E). There were no significant changes in mRNA levels of VCAM-1 and E-selectin after α -Gal A treatment (data not shown).

Steady state expression of ICAM-1 and VCAM-1 was almost undetectable with Western blot. The expression levels were markedly increased in TNF- α -treated cells. No significant difference was observed in VCAM-1 and ICAM-1 protein levels in cells with or without Gb₃ loading at both steady state or after TNF- α -stimulation (Fig. 5A). Also, there was no significant difference in ICAM-1 expression level between the EtDO-P4-treated and untreated control cells (data not shown). However, VCAM-1 protein after TNF- α -stimulation was significantly decreased in EtDO-P4-treated cells comparing with untreated cells (Fig. 5B).

Plasma from Fabry patients increases ROS production in IMFE1 cells

We had previously found that incubation of normal and Fabry vascular endothelial cells with plasma of untreated patients with Fabry disease markedly increases intracellular Gb₃ level (data not shown). The lysosomal accumulation of Gb₃ in endothelium in Fabry patients is thought to originate in circulating Gb₃ [2].

To explore the possible effects of plasma from Fabry patients on ECs under conditions that mimic *in vivo* environments, IMFE1 cells were incubated with 20% human plasma for 48 hrs and intracellular ROS production was analyzed. Fabry plasma led to significantly increased ROS production in IMFE1 cells when compared to that with non-Fabry plasma (Fig. 6A). To determine whether the increased ROS production was attributed to increased lysosomal Gb₃ uptake from patients' plasma, intracellular Gb₃ levels were evaluated by immunostaining in these cells. Although there were moderate variations in the intensities of Gb₃-positive signals among the cells incubated with individual plasma, no conclusion could be drawn regarding the effect of Fabry plasma on intracellular Gb₃ storage. Since we used for these experiments plasma from patients on long-term ERT, the effect of Fabry plasma on ROS production in IMFE1 may not be directly related to an increased endosomal content of Gb₃.

Plasma-loading study was also performed on non-Fabry primary vascular endothelial cells (HMVEC). There was no statistical significance ($P > 0.05$) in ROS generation in HMVEC incubated with Fabry and non-Fabry plasma, although there was a trend for higher ROS levels in cells incubated with patients' plasma (Fig. 6B).

Fabry plasma incubated with IMFE1 cells pretreated with EtDO-P4 caused significantly higher ROS production than non-Fabry plasma on these cells (Fig. 6C). Fabry plasma also caused higher ROS production in IMFE1 cells pretreated with recombinant α -galactosidase A to reduce Gb₃ (Fig. 6D). These data suggested that intracellular Gb₃, or other affected GSL, had no or little synergistic effect on plasma-induced ROS generation. Unexpectedly, α -galactosidase A treatment significantly increased ROS generation in the IMFE1 cells incubated with non-Fabry plasma or FBS compared with untreated cells (Fig. 6D).

Discussion

Our study showed direct evidence that accumulated Gb₃, in a dose-dependent manner, induces oxidative stress and up-regulates adhesion molecules expression in Fabry vascular endothelial cells indicating a potential mechanism for the vascular involvement in Fabry disease. Factors in patients' plasma may also contribute to the overproduction of ROS. These *in vitro* data are further supported by previous clinical findings showing excessive formation of ROS in the patients with Fabry disease [10;13;20].

Growing evidence indicate that excessive production of ROS has a causal role in the development of atherosclerosis and other cardiovascular disorders through a number of mechanisms such as oxidation of low-density lipoprotein and modification of endothelial functions and adhesion molecules [21;22]. At the cellular level, overproduced ROS damages DNA, lipids and proteins through oxidation and leads to cellular dysfunction.[23] The present study suggests that oxidative stress could participate in the pathogenesis of vascular complications in Fabry disease.

The mechanism by which elevated intracellular Gb₃ induces ROS production in vascular endothelial cells is unclear. It is possible that Gb₃ activates oxidative enzymes such as nicotinamide adenine dinucleotide (phosphate) (NADPH) oxidases the major source of ROS generation in vascular endothelial cells [23]. In addition, it has been shown that endothelial nitric oxide synthase (eNOS) is another important source of ROS generation under certain pathological conditions [24;25;26]. Superoxide generated by this mechanism (referred as eNOS uncoupling) plays an important role in endothelial dysfunction [27]. Interestingly, a recent study showed increased Gb₃ and related GSL in the caveolar fractions of endothelial cells from a mouse model of Fabry disease [28]. It is possible that altered lipid rafts in caveolae by Gb₃ accumulation causes eNOS uncoupling increased ROS generation such as superoxide.

Another important finding of the present study is that increased Gb₃ content up-regulates the expression of CAMs in vascular endothelial cells at the transcriptional level with a down-regulation of CAMs expression in response to reduced endogenous Gb₃ both by EtDO-P4 and enzyme treatment. This is consistent with findings in patients with Fabry disease [11]. It has been shown that the amount of soluble CAMs correlates directly with intracellular expression of the CAMs *in vitro* [29]. E-selectin is involved in the rolling of leukocytes, and ICAM-1 and VCAM-1 induce subsequent firm adhesion of leukocytes to endothelium [30], and its over-expression may play a role in the vasculopathy of Fabry disease.

The induction of ICAM-1, VCAM-1 and E-selectin in endothelial cells is predominantly regulated at a transcriptional level. ROS are involved in the up-regulation of endothelial CAMs through the activation of the transcription factors, mainly nuclear factor-kappa B (NF-κB) [31]. It is possible that the up-regulation of CAMs induced by Gb₃ in this study is mediated by excess ROS. Despite the increased transcription of CAMs induced by Gb₃-loading, no changes of steady state expression of these molecules could be detected by Western blot analysis. The possible explanation is the low expression level of these proteins on inactivated vascular endothelial cells is below detection limit of Western blot.

Our results suggest that intracellular Gb₃ does not have synergistic effect to patients' plasma on ROS generation because reduction of Gb₃ by EtDO-P4 and α-galactosidase A couldn't eliminate the effect of plasma. Fabry patients' plasma also increased ROS generation in normal ECs (HMVEC), although not significantly so. We speculate that certain altered circulating factors (possibly inflammatory cytokines or other GSL) in Fabry plasma resulting lead to excess production of ROS. We did not exclude that circulating globotriaosylsphingosine is responsible for some of the effects we observed [32]. The methods we used were not designed to detect the very low concentration of, in the nM range, of the more water soluble

globotriaosylsphingosine (Gb₃ level is in the μM range). Interestingly, patients with some residual α-galactosidase A activity had normal globotriaosylsphingosine levels in plasma [32].

Unexpectedly, treatment of IMFE1 cells with recombinant α-galactosidase A led to increased generation of ROS. The enzyme treatment also enhanced the TNF-α-induced increment of ICAM-1 mRNA although it was associated with decreased steady state ICAM-1 mRNA after enzyme treatment. α-Galactosidase A treatment under these *in vitro* conditions appears to enhance oxidative stress and the reactivity of the vascular endothelial cells to inflammatory cytokines suggesting that the biological effects of ERT may be more complicated than depletion of accumulated Gb₃. However, it should be noted that the observations in this study consist of “short-term” experiments. Although α-galactosidase A is specific to GSL with terminal α-D-galactosyl residues, acute catabolism of its substrates may cause excessive generation of their metabolic products and temporary imbalance in the biosynthesis and degradative pathways of other GSL. These changes in GSL may influence ROS generation and adhesion molecules expression. However, these findings may be relevant to the fact that strokes continue to occur in patients on long-term ERT [33;34;35;36].

The availability of an authentic *in vitro* model system is important for the study of disease mechanisms. IMFE1 cells were established by introduction of human telomerase and have a significantly extended lifespan. This cell line has a stable phenotype and expresses the major constitutive and inducible endothelial markers (ICAM-1, VCAM-1 and E-selectin) as shown in this study and retains functional characteristics of vascular endothelial cells [15]. The ease of manipulating Gb₃ level makes IMFE1 an ideal model to study biological effects of Gb₃ in endothelial cells. In contrast, comparing a limited number of different cell lines from Fabry patients and non-Fabry controls may be inadequate for mechanistic studies. The intrinsic differences in genetic background and endothelial characteristics of different isolates could make analysis difficult to perform.

Taken together, the present study demonstrates that Gb₃ induces ROS production and regulates transcription of CAMs in vascular endothelial cells of Fabry patients. Besides pathological conditions, our results may have implications in providing new insights to biological role of Gb₃ and/or α-galactosidase A in maintaining vascular functions in physiological conditions. Further studies of endothelial cells *in vitro*, together with animal and clinical studies would lead to better understanding of pathogenesis of Fabry disease and developing new therapeutic strategies.

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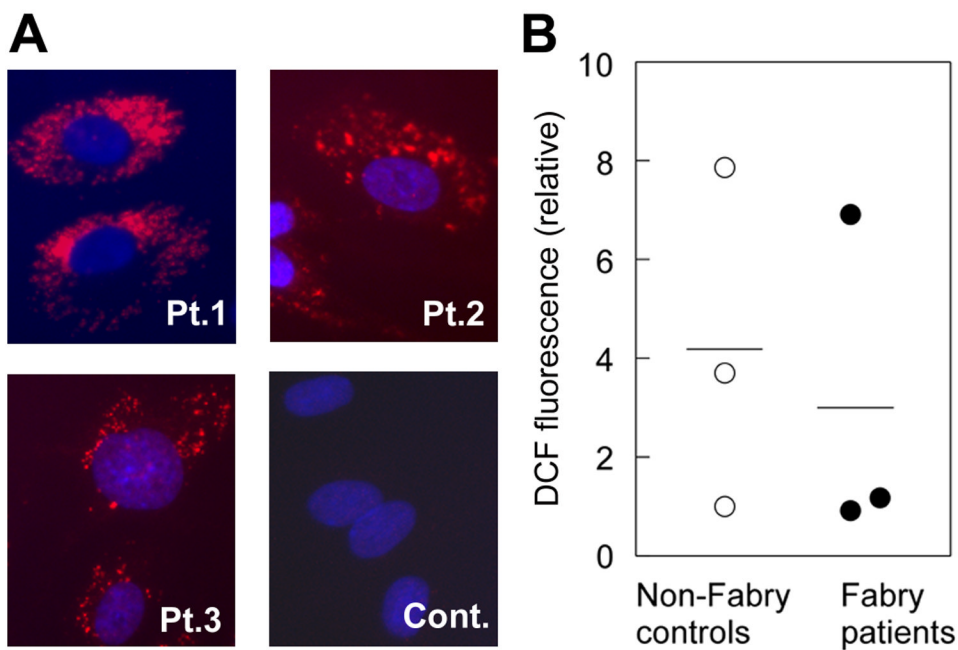


Figure 1. Assessment of ROS production in primary ECs
 (A) Immunostaining for Gb₃ in primary ECs. Significant lysosomal accumulation of Gb₃ could be detected in all 3 Fabry patients' ECs (Pt. 1–3) but not in control's ECs (cont). (B) Intracellular ROS generation in primary ECs was assessed by DCF fluorescence dye. There was no significant difference between the cells from Fabry patients and controls ($P > 0.5$, Mann-Whitney U test). The results are presented as mean of the data from six wells for each cell line. The results shown are representative of three independent experiments.

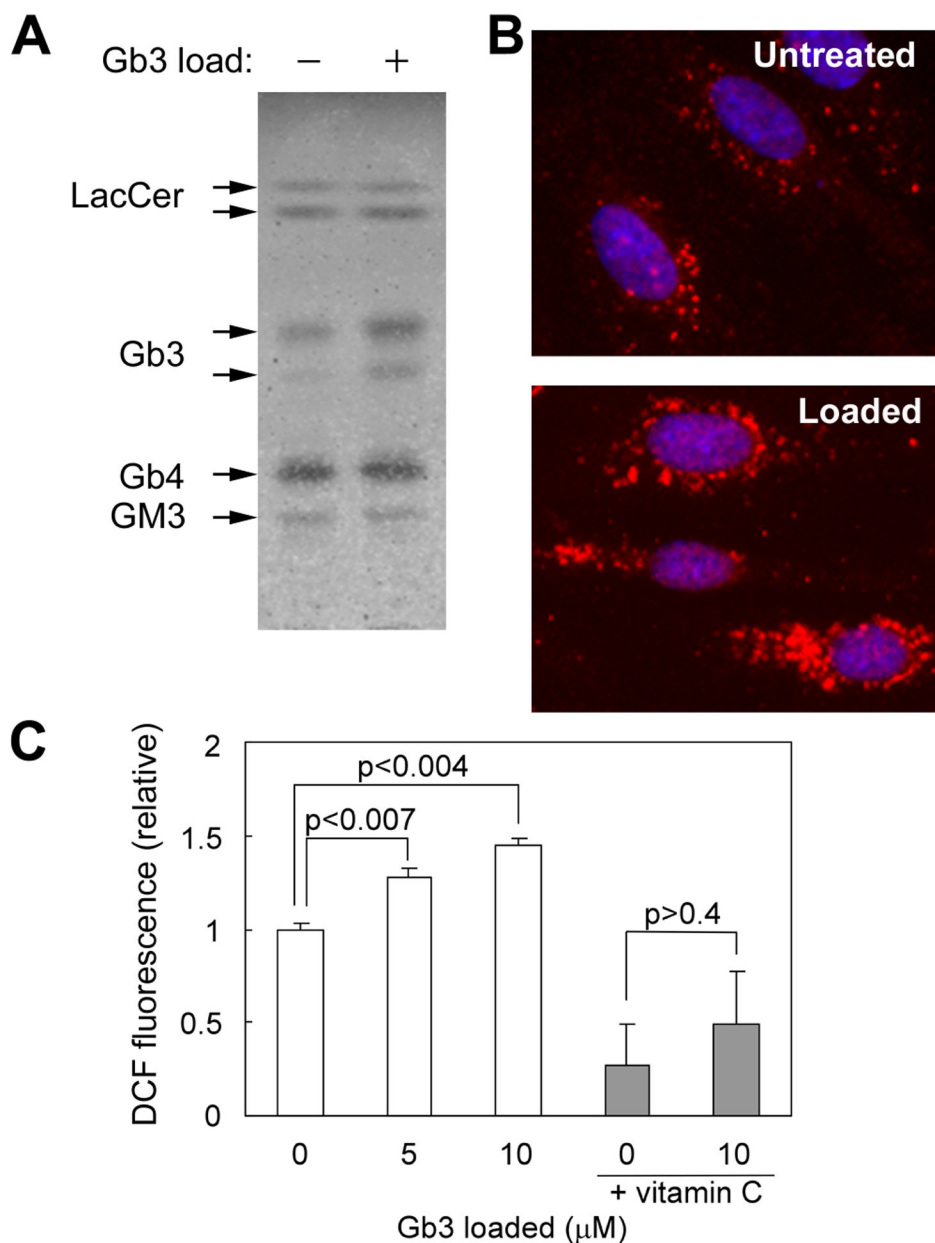


Figure 2. Effects of Gb₃ on the generation of ROS in IMFE1 cells

IMFE1 cells were incubated with the medium containing Gb₃/albumin complex for 3 days. Increase of intracellular Gb₃ was verified by TLC (A) and immunostaining against Gb₃ (B). Intracellular Gb₃ was significantly increased after incubation with Gb₃ (10 $\mu\text{mol/L}$) without significant changes in other GSLs. (C) Intracellular ROS generation was significantly increased in the cells loaded with exogenous Gb₃ (5 and 10 $\mu\text{mol/L}$). The effect on ROS production was dependent on the concentrations of Gb₃ in the media. Addition of 100 $\mu\text{mol/L}$ vitamin C into the loading media significantly lowered ROS generation in the cells and abrogated the effect of Gb₃ on ROS generation. The data are presented as mean \pm SE ($n = 6$). Mann-Whitney U test was used to compare the statistical significance. Results shown are representative of three independent experiments.

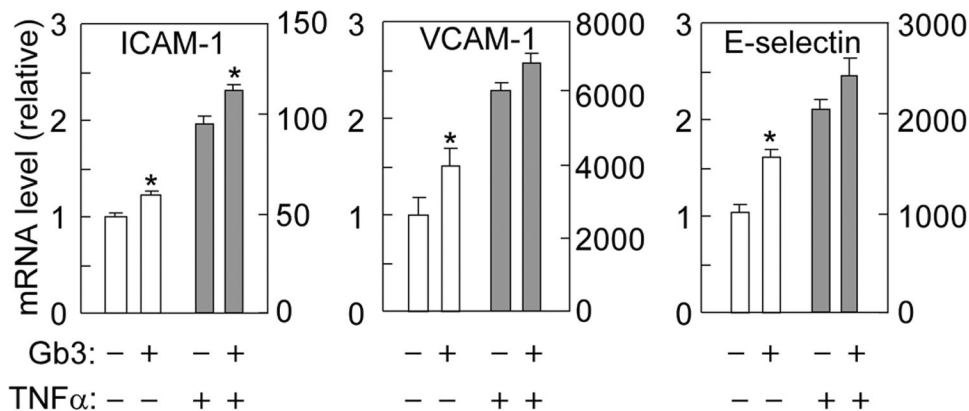


Figure 3. Effects of Gb₃ on CAMs expression in IMFE1 cells

IMFE1 cells were incubated in the medium containing Gb₃ (10 μmol/L) for 2 days, then cells were treated with or without TNF-α (100 units/ml) for 4 hours. Total RNA was extracted and mRNA levels of CAMs were analyzed by TaqMan RT-PCR. Gb₃-loading significantly increased steady state mRNA levels of all three CAMs analyzed, and increased ICAM-1 mRNA after TNF-α-stimulation. Results are obtained from three wells each and are representative of two independent experiments. The data are expressed as a ratio to untreated cells and presented as mean ± SE ($n = 3$), * $P < 0.05$, Mann-Whitney U test. Different y-axis scales are used for the absence (left) and presence (right) TNF-α treatment.

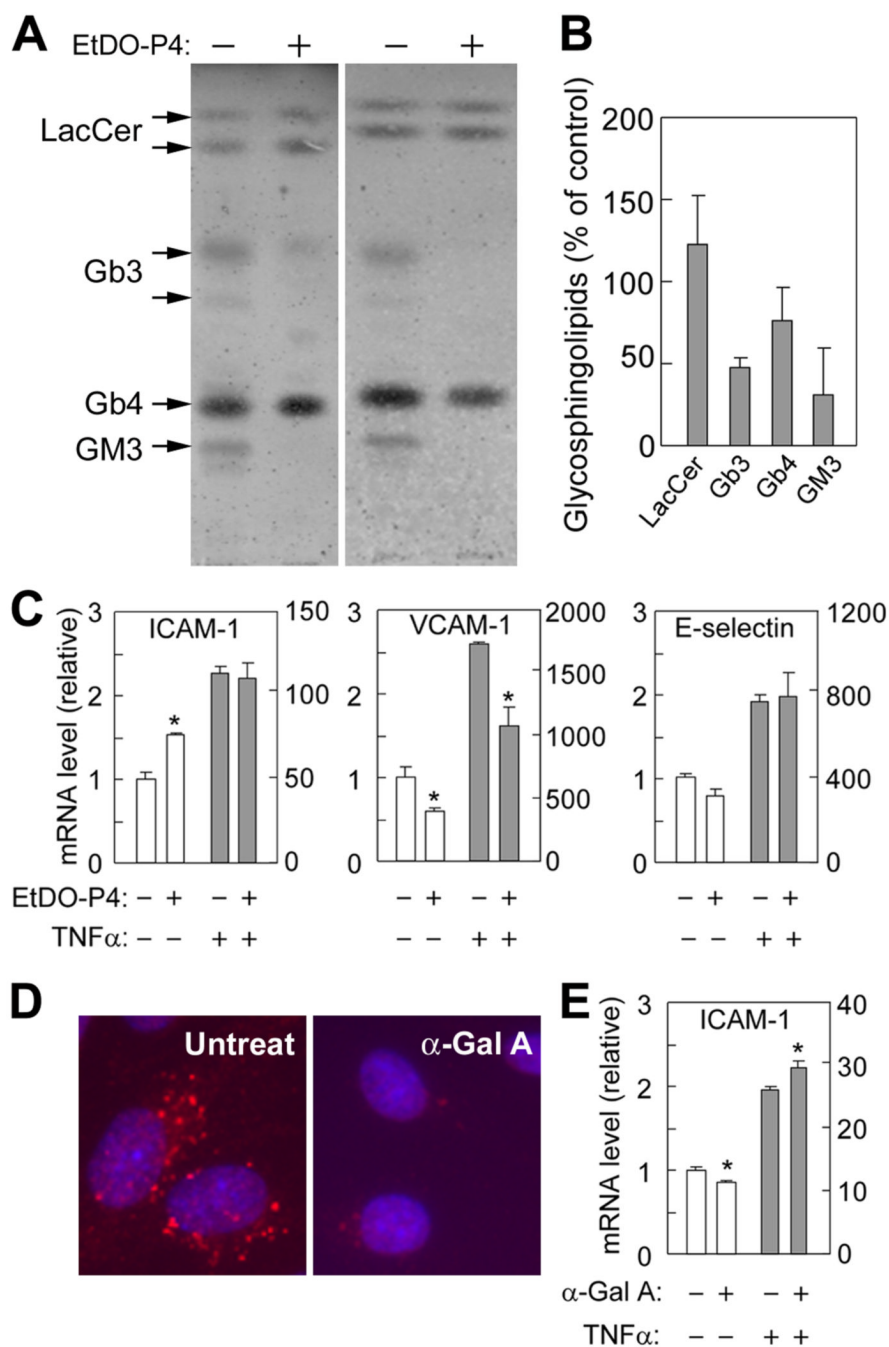


Figure 4. Effects of EtDO-P4 and α -galactosidase A (α -Gal A) treatments on CAMs expression
 IMFE1 cells were treated with 1 μ mol/L EtDO-P4 for 10–17 days (A–C) or 0.08 IU/ml recombinant α -Gal A for 4 days (D and E). (A) Reduction of Gb₃ in EtDO-P4 treated cells was verified with TLC. Graphs shown are the results of two independent experiments. (B) Quantitative data of GSLs levels obtained from the experiments shown in (A). The data are presented as mean \pm SE ($n = 2$). (C) Effect of EtDO-P4 treatment on CAMs expression in presence and absence of TNF- α stimulation. (D) Lysosomal accumulation of Gb₃ was reduced significantly by α -Gal A treatment when analyzed by immunostaining against Gb₃. (E) Effect of α -Gal A treatment on ICAM-1 expression in the presence and absence of TNF- α stimulation. Different y-axis scales are used for the absence (left) and presence (right) of TNF- α treatment.

Results are obtained from three wells and presented as mean \pm SE ($n = 3$), * $P < 0.05$, Mann-Whitney U test.

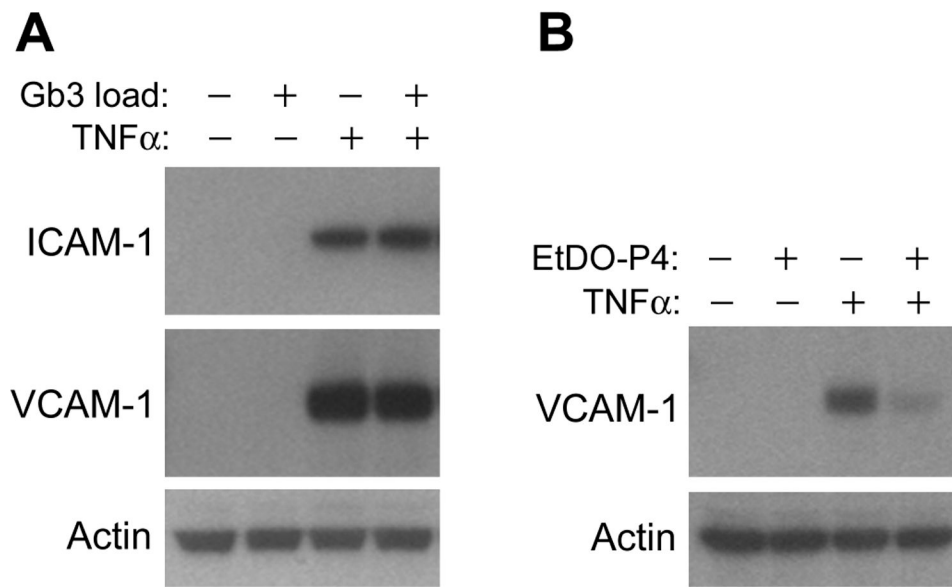


Figure 5. Western blot analysis for CAMs expression after Gb3-loading and EtDO-P4 treatment
 IMFE1 cells were incubated with 10 $\mu\text{mol/L}$ Gb3 for 2 days (A) or 1 $\mu\text{mol/L}$ EtDO-P4 for 17 days (B). The cells were treated with and without TNF- α (100 units/ml) for 6 hours before being harvested for Western blot analysis. β -actin was used as loading control. Results are representative of two independent experiments.

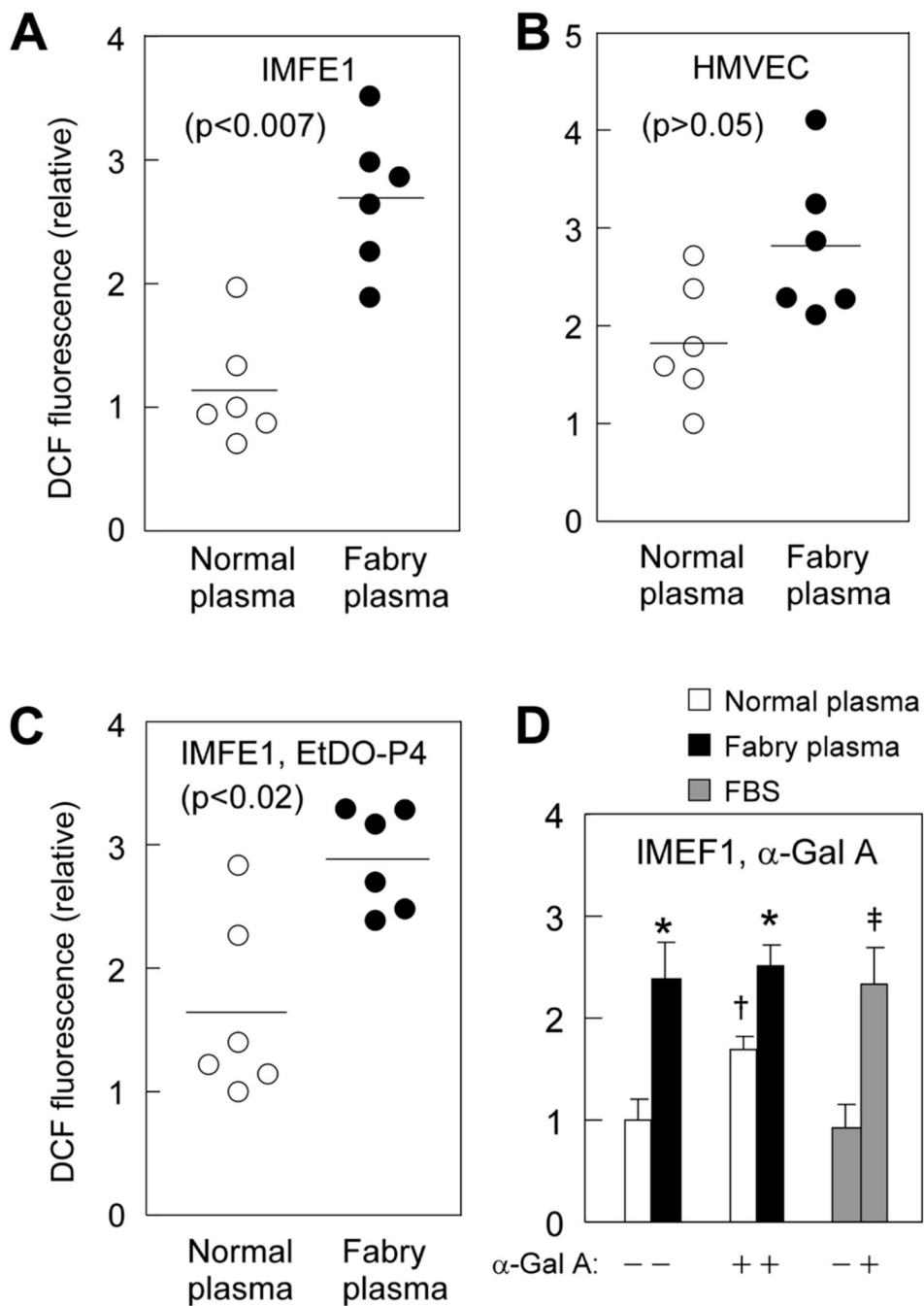


Figure 6. Effect of patients' plasma on ROS generation in ECs

Endothelial cells with or without indicated pre-treatments were incubated with 20% plasma obtained from either Fabry patients ($n = 6$) or non-Fabry controls ($n = 6$) for 2 days, and intracellular ROS generation was assessed. (A) Effect of plasma on IMFE1 cells. (B) Effect of plasma on primary normal ECs (HMVEC). (C) IMFE1 cells were treated with 1 μ M EtDO-P4 for 3 weeks to reduce endogenous Gb₃. The cells then were incubated with 20% plasma in the presence of EtDO-P4 for 2 days. (D) IMFE1 cells were incubated with 0.08 IU/ml recombinant α -Gal A for 4 days to deplete endogenous Gb₃. Then, the cells were incubated with 20% human plasma or FBS in the absence of α -Gal A for 2 days before DCF dye analysis. * $P < 0.02$, cells incubated with Fabry plasma were compared with the cells incubated with

non-Fabry plasma; † $P < 0.05$, cells treated with α -Gal A were compared with un-treated cells (both cells were incubated with non-Fabry plasma after enzyme treatment); ‡ $P < 0.03$, cells treated with α -Gal A were compared with un-treated cells (both cells were incubated with FBS after treatment). Mann-Whitney U test was used to compare for statistical significance.