BMB
reports

Globotriaosylceramide (Gb3) content in HeLa cells is correlated to Shiga toxin-induced cytotoxicity and Gb3 synthase expression

In-Sun Shin[1], Satoshi ishii[2], Jong-Seo Shin[1], Kyong-Il Sung[1], Byung-Sung Park[1], Hyun-Yong Jang[1] & Byong-Wan Kim[1,]*

[1]College of animal Life Sciences, Kangwon National University, Kangwon 200-701, Korea, [2]Department of Agriculture and Life Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan

Globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4) are the proposed functional receptors for Shiga toxin (Stx). To elucidate the effect of Gb3 content on Stx-induced cytotoxicity in HeLa cells, we cloned HeLa cells and determined the correlation between glycolipids content and Stx-induced cytotoxicity. The 29 HeLa cell clone (HLCC) lines used showed a wide range of sensitivity to Stx, compared to Gb3-rich cells which were more sensitive, showing as little as 20% viability to 100 pg/ml Stx. In contrast, Gb3-deficient cells proved resistant as they were more than 80% viable to 100 ng/ml Stx. Gb3 content in the HLCC lines corresponded with Stxs-induced cytotoxicity as well as Gb3 synthase expression, but no correlation with Gb4 content was noted. These data show that Gb3 content, which is regulated by the expression of Gb3 synthase, determines the sensitivity of HeLa cells toward Stx. [BMB reports 2009; 42(5): 310-314]

INTRODUCTION

Shiga toxins (Stxs, also referred to as verotoxins), a family of bacterial protein toxins generated by Stx producing-*Escherichia coli* (STEC) such as *E. coli* serotype O157:H7, are believed to cause hemorrhagic colitis and hemolytic uremic syndrome (HUS) (1, 2). Stxs are divided into two main groups referred to as Stx1 and Stx2. Stx1 is essentially identical to Stx from *Shigella dysenteria*, whereas Stx2 is approximately 56% homologous in amino acid sequence with Stx1 (3-5). The results of epidemiological and experimental studies have indicated Stx2 may be of greater clinical significance than Stx1 (6, 7).

All shiga toxins consist of a single A subunit in association with several pentamer B subunits (8-10). The A subunit as a ribosomal RNA *N*-glycosidase removes the adenine of the 28S RNA within the 60S ribosomal subunit (11), thereby rendering ribosomes inactive for protein synthesis (12). In contrast, functionality of the B subunit requires binding to the plasma membrane and internalization (4). The B subunit of these toxins are 62% identical in amino acid sequence (9), and moreover bind specifically to the glycosphingolipid Gb3, which is present on select eukaryotic cells (13, 14). Stxs have further been reported to bind to globotetraosylceramide (Gb4), which harbors an *N*-acetyl-D-galactosamine residue that connects to the trisaccharide of Gb3 (15, 16). The binding of Stx2 to Gb4 apparently explains the preferential cytotoxicity for Vero and HeLa cells (17, 18).

In a recent report Gb3 synthase knock-out mice exhibited a loss of sensitivity to verotoxins following the complete deletion of globo-series glycosphingolipids (19). However, the functional receptor that mediates Stx cytotoxicity remains unknown because it is unclear which of the two glycolipid receptors, either Gb3 or Gb4, is the principal factor mediating the observed loss of sensitivity to verotoxins.

In this study, we cloned HeLa cells and analyzed their cytotoxicity to Stxs in order to determine the correlation between Gb3 and Gb4 content and Stx-induced cytotoxicity.

RESULTS

Gb3 content in HeLa cell clones

In this study 29 HLCC lines were obtained from the parental HeLa strain cell line by performing limiting dilutions. Basal levels of Gb3 and Gb4 content were assessed via several different techniques. The pattern of glycosphingolipid expression was analyzed by TLC and revealed different levels of Gb3 and Gb4. However the content of LacCer and GlcCer was almost the same (Fig. 1). Quantification of Gb3 and Gb4 revealed greater variation in Gb3 content (1.2-12.9 μg/mg protein) than in Gb4 (1.2-5.6 μg/mg protein). Varying levels of Gb3 were also observed when tested by flow cytometry (data not shown).

Differential sensitivity of HeLa cell clones to Shiga toxins

In order to evaluate the cytotoxicity of Stxs in HLCC lines, various dilutions of Stx1 (1 pg-100 ng/ml) were prepared and incubated with cell monolayers, followed by determining the number of remaining viable cells by MTT assay. A total of 29 HLCC were acquired, including five highly-sensitive clones

*Corresponding author. Tel: 82-33-250-8625; Fax: 82-33-251-7719; E-mail: bwkim@kangwon.ac.kr

Received 8 October 2008, Accepted 22 February 2009

Keywords: Gb3 content, Gb3 synthase expression, Glycolipids, HeLa cells, Shiga toxin

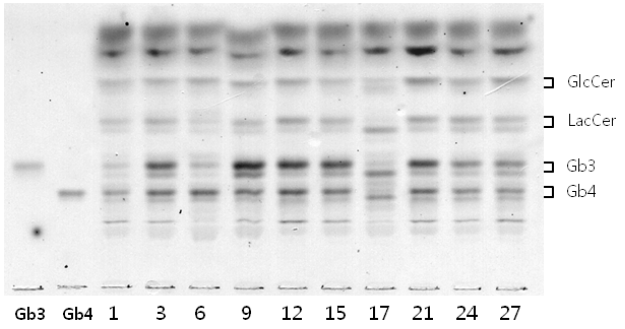

Fig. 1. TLC of glycosphingolipids extracted from HLCC lines. Cellular lipids were extracted from 1-2 × 10^6 cell with chloroform/methanol (2:1), and Folch's lower fractions that correspond to 0.12 mg cell protein were separated on TLC plates in a chloroform/methanol/water (60:35:8) solvent system. Glycosphingolipids were visualized by spraying orcinol-sulfuric acid reagent. 200 ng each of the standard glycosphigolipids of Gb3 and Gb4 were separated on a TLC plate simultaneously and the position of each lipid is indicated.

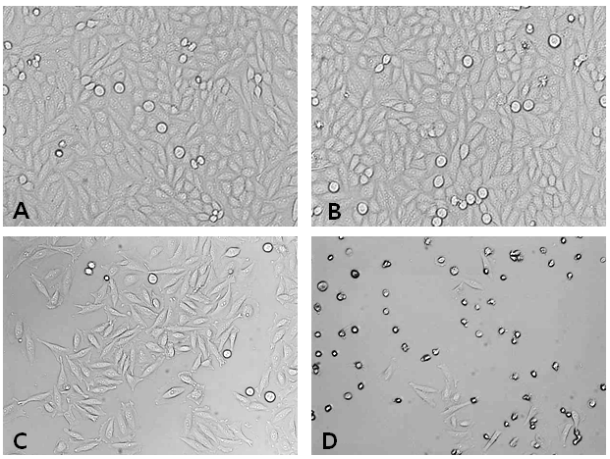

Fig. 2. Morphology of Stx-treated HeLa cells. HeLa cell clones were obtained from the parental strain HeLa cell line by limiting dilutions. HLCC1 (A) and HLCC12 (B) (each 1 × 10^4) was incubated in a 96-well plate with Stx1 (100 pg/ml) for 24 hr. Phase-contrast micrographs of HLCC1 (C) and HLCC12 (D) are showed. Original magnification ×100.

with minimum CD_{50} (lower than 10 pg/ml Stx) values, and six low-sensitivity clones with an undetectable CD_{50} (higher than 100 ng/ml Stx) values (data not shown). HLCC lines, which demonstrated considerably different levels of Gb3, were tested for viability in the presence of Stx1 (Fig. 2). Results reveal viability of the cell clones differs as levels of Gb3 range from deficient to rich. Cellular viability was reduced markedly upon increased levels of Stx1, and the HLCC lines evidenced a wide range of sensitivity. Gb3-rich cells (HLCC12) were more sensitive, showing as little as 20% viability to 100 pg/ml Stx. In contrast, Gb3-deficient cells (HLCC1) proved resistant,

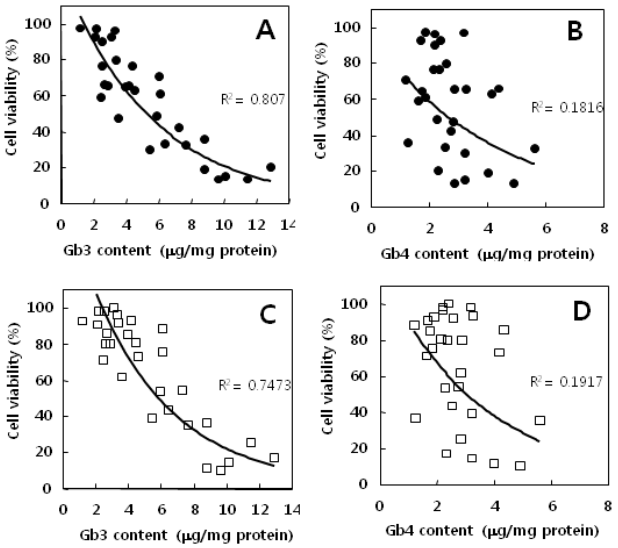

Fig. 3. Correlation of Gb3, Gb4 content and cell viability in HLCC lines. Gb3 and Gb4 cell content in whole lines of HLCC was determined as described in "materials and methods". Cell viability in response to 100 pg/ml of Stx1 was determined by MTT assay, and was compared to quantified levels of Gb3 (A) and Gb4 (B). Also presented is the viability in response to Stx2, and was compared to levels of Gb3 (C) and Gb4 (D). The solid line represents the best fit of the data to a negative exponential equation.

showing more than 80% viability to 100 ng/ml Stx. Therefore, these two lines were utilized in order to assess the morphological changes induced by Stx. Eight hours following treatment of the two cell lines with Stx1 (100 pg/ml), cells rich in Gb3 (HLCC12) became apoptotic and displayed cytoplasmic shrinkage. After 24 hours of incubation they were released in the culture medium, whereas no such morphological changes were observed in the Gb3-deficient cells (HLCC1).

Correlation of Gb3, Gb4 content and cell viability to Shiga toxins

Cellular viability in the presence of Stx1 (100 pg/ml) was inversely related to Gb3 content, as high levels of Gb3 were detected in relatively low-viability cases (less than 20%) and low levels of Gb3 were observed in relatively high-viability cases (more than 80%). This is a high level of correlation with an R^2 value of 0.807 (Fig. 3A). In contrast, no such correlation was observed between cellular viability and Gb4 content (Fig. 3B). A comparison with Stx2 further revealed the correlation between cell viability and levels of either Gb3 (R^2=0.7473) or Gb4 was fairly similar to that of Stx1 (Fig. 3C, D).

Expression level of Gb3 synthase mRNA

To investigate the differing levels of Gb3 within the HLCC lines, the expression levels of enzymes that affect Gb3 content were assessed. The differential levels of α1,4Gal-T (Gb3 synthase) expression were observed, but the expression level of

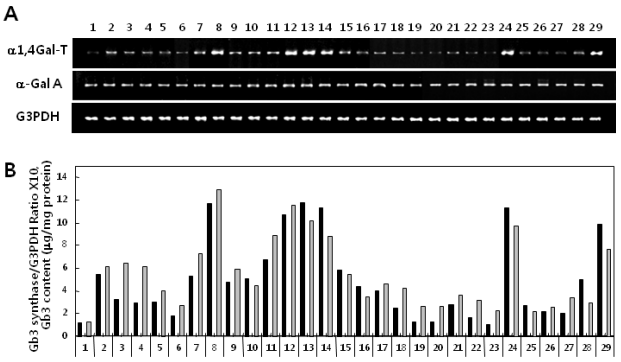

Fig. 4. Expression level of Gb3 metabolic enzymes in HLCC lines. RNAs were prepared from whole line of HLCC, and RT-PCR was performed for Gb3 synthase, α-Gal A and G3PDH expression. (A) Representative analysis of RT-PCR products. (B) The bands were quantified and presented along with the relative optical density of the Gb3 synthase PCR signal, which is normalized to the PCR signal of G3PDH (black bars) and Gb3 content (gray bars).

α-galactosidase A, which catabolizes Gb3 to LacCer, was almost identical amount in all HLCC lines (Fig. 4A). The Gb3 synthase/G3PDH ratio was approximately 10-fold greater in Gb3-rich cells (1.13) than in Gb3-poor cells (0.12), which is similar to the observed pattern of Gb3 content (Fig. 4B). In addition, our results demonstrate the expression level of Gb3 synthase corresponds well to Gb3 content, as evidenced by a R^2 value of 0.7602 (data not shown).

DISCUSSION

In this study, 29 HLCC lines were obtained from the parental HeLa cell strain via limiting dilution, and the basal levels of Gb3 and Gb4 were measured using several different methods. The pattern of glycosphingolipid expression was analyzed via TLC and revealed differences in Gb3 content among the HLCC lines. However the content of LacCer or GlcCer were almost identical. The HLCC lines displayed a wide range of sensitivity to Stx. Cell viability upon exposure to Stx is inversely proportional to Gb3 content, as high levels of Gb3 are present in low-viability cases, and low levels of Gb3 are seen in high-viability cases. Therefore these data indicate the Gb3 content of HeLa cells determines the cytotoxic response to Stxs. The high sensitivity of Gb3-rich cells toward Stxs may be may be attributable to apoptosis, as reported by Fujii *et al*. (20).

Differences in cytotoxicity of Stx in the cloned cell lines have been described in several previous studies (21, 22). Accordingly, HeLa cells were cloned in this study and the correlation between Gb3 or Gb4 content and cell viability upon exposure to Stxs was assessed. In order to exclude the experimental artifacts due to unusual conditions, we assessed the cytotoxicity of Stx using all 29 HLCC lines, which consequently have different levels of Gb3. The length of fatty acids has been previously shown to affect the mobility of glycolipids in TLC (23). In this study, these findings were also observed in HLCC 17. However, differences in sensitivity in HLCC 17 and other lines with the same level of Gb3 were not observed. In addition, the role of lipid rafts in signaling has been reported, as they function as physical platforms for the concentration and assembly of signal transduction machinery (24-26). Thus, the low-viability Gb3-rich cells upon exposure to Stxs may be a consequence of a membrane rich in lipid rafts, and this result would bolster the conclusions of previous studies (27, 28).

In an effort to elucidate the regulatory mechanisms underlying Gb3 synthesis in the HLCC lines, the expression levels of enzymes associated with Gb3 metabolism were assessed. While differential levels of α1,4Gal-T (Gb3 synthase) expression were absorbed, α-galactosidase A displayed almost identical expression levels in all HLCC lines. The expression of Gb3 on the cellular surface was assessed via flow cytometry with anti-Gb3 mAb and found clone with high levels of Gb3 synthase displayed stronger fluorescence than the low expression clone (data not shown). In addition, the expression level of Gb3 synthase corresponds well to Gb3 content. These data show the level of Gb3 synthase expression determines the level of Gb3 in HeLa cells.

In an effort to understand the upregulation of Gb3 synthase, we evaluated the idea that cytokines could elicit secretion of TNF-α and IL-1, which would then bind to HeLa cells and induce expression of Gb3 via the well-established phenomenon of receptor-mediated Gb3 expression (29, 30). However, neither TNF-α nor IL-1 mRNA was observed to have a relationship with Gb3 content in our HLCC lines (data not shown). It was reported the exact mechanism of Gb3 synthase activation was unknown, however the results of the present study support the notion that a pathway of Gb3 expression in HeLa cells exists, and will require further study for its thorough elucidation.

In conclusion, our results demonstrate that the level of Gb3, which determines sensitivity toward Stxs, may be regulated in HeLa cells via the expression of Gb3 synthase. In addition, these cell lines should provide further insights into the molecular mechanisms underlying Gb3 expression relative to cytotoxic action, and further understanding of cell injury mediated by bacterial toxins, possibly in the development of a screening method that could protect against Stx-mediated cytotoxicity.

MATERIALS AND METHODS

Materials

Stx1 and Stx2 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). HisProbe-HRP was purchased from Pierce Biotechnology, Inc. (Rockford, IL). All other chemical reagents were acquired from Sigma-Aldrich Fine Chemical (St. Louis, Mo., USA.), unless otherwise indicated.

Cloning and maintenance of HeLa cells

The HeLa cell line (JCRB9004) was purchased from the Hu-

man Science Research Resources Bank (Osaka, Japan) and maintained at 37°C in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated (55°C, 30 min) fetal calf serum (Biological Industries, Haemek, Israel) under a humidified 5% CO_2 atmosphere. Pre-cultured cells were cloned by limiting dilutions (21). The HeLa monolayers were disaggregated in trypsin-EDTA (Invitrogen Japan K.K., Tokyo) and counted and diluted to 10 cell/ml in culture medium. Of this diluted cell suspension, 100 μl was seeded onto 96-well plates. Colonies that developed over 2 weeks were serially transferred to 12-well plates and then to 6-well and 10-cm culture dishes. Gb3 content of the HeLa cell clone (HLCC) was assessed in terms of its sensitivity to Stxs. Cells were recultured, retested and maintained via weekly passage. Phenotypic consistency was confirmed weekly over the course of these experiments.

Glycosphingolipid extraction

Glycosphingolipids were extracted from HeLa cells grown in 10-cm culture dishes. Cultured HLCC lines ($1\text{-}2\times10^6$) were washed in PBS, scraped and precipitated after 5 min of centrifugation at 2,000 rpm. Cell pellets were homogenized with a micro homogenizer (Physcotron, Niti-on, Inc., Chiba, Japan) in 0.2-ml of H_2O. Following the determination of protein content with a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA), the cellular lipids were extracted from homogenates containing 5-10 mg of protein with 20 volumes of chloroform-methanol (2:1, v/v). After filtration using 90- mm 5A filter papers (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), total extracts were dried and further treated for 2 h with 1 ml of methanolic NaOH (0.2 M NaOH in methanol) at 40°C. After neutralizing the solution with glacial acetic acid, the lipids were further subjected to Folch's partitioning (chloroform-methanol-H_2O, 8:4:3 in v/v/v) (31). Glycosphingolipids recovered in the lower phase were pooled and dried, and the Folch's lower fractions were utilized as a sample of cellular glycosphingolipids.

High-performance thin-layer chromatography (HPTLC)

The entire content of glycosphingolipids was extracted from $1\text{-}2\times10^6$ of HLCC lines as described above, and were separated on a HPTLC-Silica gel 60 plates (Merck & Co., Inc., Whitehouse Station, NJ) in a chloroform-methanol-water (60:35:8, v/v/v) solvent system. Glycosphingolipids were visualized by spraying orcinol-sulfuric acid reagent for 5-10 min at 100°C as previously described (32).

Quantification of Gb3 and Gb4

The content of Gb3 in the HLCC lines was determined via 1B-His binding assay, as described in our previous study [32]. In order to determine Gb4 content, TLC analysis was conducted quantitatively with TLC plates using a solvent system of chloroform-methanol-water (60:35:8, v/v/v). Glycosphingolipids were visualized by spraying with orcinol-sulfuric acid reagent; and the Gb4 band intensities were determined using Scion Image image processing software (Scion Corporation, Frederick, MD).

Cell viability assay

HLCC lines were plated at 1×10^4 cells/well in 96-well plates, then allowed to settle via overnight cultivation. Stx1 and Stx2 were diluted to 100 pg/ml in culture medium, and 100 μl of the dilutions were transferred to the cells. After 48 h of incubation, viable cell counts were estimated using an MTT In Vitro Toxicology Assay Kit (Sigma, St. Louis, Mo., USA.), in accordance with the manufacturer's recommendations. Absorbance was measured at 570 nm and the cell viability was expressed as a percentage of the untreated cell count.

Reverse transcription-PCR (RT-PCR) analysis

For RT-PCR, total RNA was extracted using RNAiso (Takara Biotech. Co., Otsu, Japan) in accordance with the supplier's protocols. The cDNA was synthesized using an RNA PCR Kit (Takara) by incubating incubation 500 μg of cellular RNA in a 10 μl reaction mixture containing 5 mM $MgCl_2$, 2.5 μM of a Random 9-mer Primer, 1 mM each deoxynucleoside triphosphate, RNase inhibitor and AMV Reverse Transcriptase for 15 min at 42°C. PCR was conducted using *TaKaRa Ex Taq* DNA polymerase in accordance with the supplier's recommendations. cDNA was utilized as a template for amplifying the coding region of the gene using specific primers as follows: for Gb3 synthase, forward primer 5'-TTCTTCCTGGAGACTT CAGA- 3' and reverse primer 5'-CGCAGGTTCTTGAGAAC AAT-3'; for α-galactosidase A, forward primer 5'-GTCCTTGGC CCTGAATAG-3' and reverse primer 5'-TCATTCCAACCCCC TGGT-3', and for the control amplimer, Glyceraldehyde 3-Phosphate Dehydrogenase (G3PDH) (Takara), forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-T CCACCACCCTGTTGCTGTA-3'. Sizes of the PCR products for Gb3 synthase, <-galactosidase A and G3PDH were determined to be 368 bp, 231 bp and 452 bp, respectively.

REFERENCES

1. Riley, L. W., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., Hebert, R. J., Olcott, E. S., Johnson, L. M., Hargett, N. T., Blake, P. A. and Cohen, M. L. (1983) Hemorrhagic colitis associated with a rare Escherichia coli serotype. *N. Engl. J. Med.* **308**, 681-685.
2. Robson, W. L. M., Leung, A. K. C. and Kaplan, B. S. (1993) Hemolytic-uremic syndrome. *Curr. Probl. Pediatr.* **23**, 16-33.
3. Kozlov, Y. V., Kabishev, A. A., Lukyanov, E. V. and Bayev, A. A. (1988) The primary structure of the operons coding for Shigella dysenteriae toxin and temperature phage H30 shiga-like toxin. *Gene* **67**, 213-221.
4. Strockbine, N. A., Marques, L. R. M., Newland, J. W., Smith, H. W., Holmes, R. K. and O'Brien, A. D. (1986) Two toxin-converting phages from Escherichia coli O157: H7 strain 933 encode antigenically distinct toxins with similar biologic activities. *Infect. Immun.* **53**, 135-140.

5. Jackson, M. P, Neill, R. J., O'Brien, A. D., Holmes, R. K. and Newland, J. W. (1987) Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxine II encoded by bacteriophages form Escherichia coli 933. *FEMS Microbiol. Lett*. **44**, 109-114.
6. Tesh, V. L., Burris, J. A., Owens, J. W., Gordon, V. M., Wadolkowski, E. A., O'Brien, A. D. and Samuel, J. E. (1993) Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immun*. **61**, 3392-3402.
7. Marques, L. R. M., Peiris, J. S. M., Cryz, S. J. and O'Brien A. D. (1987) Escherichia coli strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II. *FEMS Microbiol. Lett*. **44**, 33-38.
8. Jacewicz, M., Clausen, H., Nudelman, E., Donohue-Rolfe, A. and Keusch, G.T. (1986) Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin- binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. *J. Exp. Med*. **163**, 1391-1404.
9. O'Brien, A. D. and Holmes, R. K. (1987) Shiga and Shiga-like toxins. *Microbiol. Rev*. **51**, 206-220.
10. Tesh, V.L. and O'Brien, A.D. (1991) The pathogenic mechanisms of Shiga Toxin and the Shiga-like toxin. *Mol. Microbiol*. **5**, 1817-1822.
11. Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T. and Igarashi, K. (1988) Site of action of a Vero toxin (VT2) from Escherichia coli O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *Eur. J. Biochem*. **171**, 45-50.
12. Obrig, T. G., Moran, T. P. and Brown. J. E. (1987) The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. *Biochem. J*. **244**, 287-294.
13. Lingwood, C. A., Law, H., Richardson, S., Petric, M., Brunton, J. L., De Grandis, S. and Karmali, M. (1987) Glycolipid binding of purified and recombinant Escherichia coli produced verotoxin *in vitro*. *J. Biol. Chem*. **262**, 8834- 8839.
14. Lingwood, C. A. (1996) Role of verotoxin receptors in pathogenesis. *Trends. Microbiol*. **4**, 147-153.
15. Waddell, T., Head, S., Petric, M., Cohen, A. and Lingwood, C. (1988) Globotriosyl ceramide is specifically recognized by the Escherichia coli verocytotoxin 2. *Biochem. Biophys. Res. Commun*. **152**, 674-679.
16. DeGrandis, S., Law, H., Brunton, J., Gyles, C. and Lingwood, C. (1989) Globotetraosylceramide is recognized by the pig edema disease toxin. *J. Biol. Chem*. **264**, 12520-12525.
17. Samuel, J. E., Perera, L. P., Ward, S., O'Brien, A. D., Ginsburg, V. and Krivan, H.C. (1990) Comparison of the glycolipid receptor specificities of Shiga-like toxin type II and Shiga-like toxin type II variants. *Infect. Immun*. **58**, 611-618.
18. Ostroff, S. M., Tarr, P. I., Neill, M. A., Lewis, J. H., Hargrett-Bean, N. and Kobayashi, J.M. (1989) Toxin genotypes and plasmid profiles as determinants of systemic sequelae in Escherichia coli O157:H7 infections. *J. Infect. Dis*. **160**, 994-998.
19. Okuda, T., Tokuda, N., Numata, S., Ito, M., Ohta, M., Kawamura, K., Wiels, J., Urano, T., Tajima, O., Fukukawa, K. and Furukawa, K. (2006) Targeted disruption of Gb3/CD77 synthase gene resulted in the complete deletion of globo-series gycosphingolipids and loss of sensitivity to verotoxins. *J. Biol. Chem*. **281**, 10230-10235.
20. Fujii, J., Matsui, T., Heatherly, D. P., Schlegel, K. H., Lobo, P. I., Yutsudo, T., Ciraolo, G. M., Morris, R. E. and Obrig, T. (2003) Rapid apoptosis induced by Shiga toxin in HeLa cells. *Infect. Immun*. **71**, 2724-2735.
21. Jacewicz, M., Feldman, H. A., Donohue-Rolfe, A., Balasubramanian, K. A. and Keusch, G. T. (1989) Pathogenesis of Shigella diarrhea. XIV. Analysis of Shiga toxin receptor on cloned HeLa cells. *J. Infect. Dis*. **159**, 881-889.
22. Sekino, T., Kiyokawa, N., Taguchi, T., Takenouchi, H., Matsui, J., Tang, W.R., Suzuki, T., Nakajima, H., Saito, M., Ohmi, K., Katagiri, Y.U., Okita, H., Nakao, H., Takeda, T. and Fujimoto, J. (2004) Characterization of a Shiga-toxin 1-resistant stock of vero cells. *Microbiol. Immunol*. **48**, 377-387.
23. Kiarach, A., Boyd, B. and Lingwood, C.A. (1994) Glycosphingolipid receptor function is modified by fatty acid content. Verotoxin 1 and Verotoxin 2c preferentially recognize different globotriaosyl ceramide fatty acid homologues. *J. Biol. Chem*. **269**, 11138-11146.
24. Carpenter, G. (2000) The EGF receptor: a nexus for trafficking and signaling. *Bioessays* **22**, 697-707.
25. McPherson, P. S., Kay, B. K. and Hussain, N. K. (2001) Signaling on the endocytic pathway. *Traffic*. **2**, 375-384.
26. Pralle, A., Keller, P., Florin, E. L., Simons, K. and Horber, J. K. (2000) Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. *J. Cell Biol*. **148**, 997-1008.
27. Pudymaitis, A., Armstrong, G.. and Lingwood, C. A. (1991) Verotoxin-resistant cell clones are deficient in the glycolipid globotriaosylceramide: differential basis of phenotype. *Arch. Biochem. Biophys*. **286**, 448-452.
28. Kongmuang, U., Honda, T. and Miwatani, T. (1998) Isolation of Shiga toxin-resistant Vero cells and their use for easy identification of the toxin. *Infect. Immun*. 56, 2491-2494.
29. Ashkenazi, A. and V. M. (1998) Dixit. Death receptors: signaling and modulation. *Scinece* **281**, 1305-1308.
30. Stricklett, P. K., Hughes, A. K., Ergonul, Z. and Kohan D. E. (2002) Molecular basis for up-regulation by inflammatory cytokines of Shiga toxin 1 cytotoxicity and globotriaosylceramide expression. *J. Infect. Dis*. **186**, 976-982.
31. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem*. **226**, 497-509.
32. Shin, I. S, Nishikawa, K., Maruyama, H. and Ishii, S. (2006) Histidine-tagged Shiga toxin B subunit binding assay: simple and specific determination of Gb3 content in mammalian cells. *Chem. Pharm. Bull*. **54**, 522-527.