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Expression of Neutral Glycosphingolipids in Cytokine-Stimulated Human Endothelial Cells

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Abstract—We compared immunohistochemical distribution of glycosphingolipids globotriosylceramide ($GbOse_3Cer$) and globotetraosylceramide ($GbOse_4Cer$) with that of E-selectin on human umbilical vein endothelial cells (HUVEC) stimulated with tumor necrosis factor (TNF)-α. HUVECs activated by TNF-α were characterized by the highest expression of E-selectin and greatest adhesion of HL-60 cells as well compared to stimulation with interleukin-1β or lipopolysaccharide. HUVECs activated by TNF-α also stained intensely with globoside antibodies, especially with the $GbOse_3Cer$-directed one, staining being redistributed in a concentration-dependent manner. These results indicate the possible role of $GbOse_3Cer$ and $GbOse_4Cer$ in immune effector mechanisms of endothelium such as adhesion.

Key words: endothelium, glycosphingolipids, E-selectin, TNF-α, immunohistochemistry

Hemostasis and inflammatory/immune reactions involve close interactions between immunocompetent cells and vascular endothelium [1]. Leukocyte migration into lymphatic tissues or inflammatory sites is regulated by the expression of adhesion and signaling molecules on leucocyte membranes and on the membranes of endothelial cells [2]. Selectins are a family of three proteins, i.e., L (leukocyte)-, P (platelet)-, and E (endothelial)-selectins, which mediate adhesive interactions between leukocytes and endothelium [3]. E-Selectin is transiently synthesized and expressed on cytokine-activated endothelial cells [3]. Interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and lipopolysaccharides (LPS) stimulate the expression of E-selectin on endothelial cells, the latter dramatically increasing the adhesion of isolated blood neutrophils [3].

Abbreviations: HUVEC) human umbilical vein endothelial cells; HL-60) human leukemia cell line; TNF-α) tumor necrosis factor α; VT-1) verocytotoxin-1; $GbOse_3Cer$ and $GbOse_4Cer$) globosides globotriosylceramide and globotetraosylceramide; LPS) lipopolysaccharides; IL-1) interleukin-1; DTAF) dichlorotriazinylaminofluorescein; DAPI) 4,6-diamine-2-phenylindole-dihydrochloride.

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Binding of selectins to cell-surface carbohydrate ligands allows flowing leukocytes to attach and then roll on the vessel wall. Selectins bind to sialylated, fucosylated, or, in some cases, sulfated glycans within glycoproteins, glycolipids, or proteoglycans [3]. Glycosphingolipid (GSL) oligosaccharide chains spread out on the cell surface, being excellent candidates for cell surface and cell–cell recognition molecules [4]. GSLs are ubiquitous and highly conserved membrane components with important biological roles in cell surface recognition [5] and in modulation of function of a variety of membrane-associated proteins [6]. GSLs are assembled as "rafts" or "glyco-signaling domains" in the outer leaflet of the plasma membrane and these clustered rather than non-clustered GSLs are biologically active [7, 8]. Cell surface GSLs may be specifically modulated by cytokines that activate endothelial cells [9, 10]; this suggests that GSLs may play a role in adhesive and/or receptor properties of activated endothelial cells.

Müthing et al. recently reported a detailed quantitative analysis of GSL distribution in human umbilical vein endothelial cells (HUVECs), a common *in vitro* model for studying the adhesion of leukocytes to endothelial cells [4]. Globotriosylceramide ($GbOse_3Cer$) and globotetraosylceramide ($GbOse_4Cer$) were the most

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abundant neutral GSLs with 36 and 23% of total staining on orcinol-treated thin-layer chromatograms (TLC) [4]. They also revealed by immunohistology the presence of GSLs in cell membrane and in cytoplasm of HUVECs [4]. Although a role of GSLs in cell adhesion is generally accepted, only a few studies on the expression of GSLs in endothelial cells have been carried out [9, 10]. Gillard et al. [9] found that activation of endothelial cells with γ-interferon resulted in a small change in GSL composition but greatly increased surface expression of gangliosides and decreased surface expression of neutral GSLs. Van de Kar et al. [10] found that preincubation of HUVECs with TNF-α resulted in a 10- to 100-fold increase in specific binding sites for verocytotoxin-1 (VT-1). Moreover, an increase of $GbOse_3Cer$, a functional receptor for VT-1, has been observed in glycolipid extracts of TNF-α-treated cells, the preincubation of human endothelial cells with TNF-α thought to lead to increase in $GbOse_3Cer$ synthesis in these cells [10]. The aim of our study was to analyze GSL expression on human umbilical endothelial cells in relation to cytokine-induced activation.

MATERIALS AND METHODS

Cells and culture conditions. The isolation of HUVECs and preparation of cell culture were described in detail elsewhere [4]. HUVECs were maintained in a 1 : 1 mixture of Iscove's MDM and Ham's F12 basal medium (Gibco BRL, Germany) supplemented with 20% (v/v) human serum (German Red Cross Blood Transfusion Service, Germany), 1.25 μg/liter human basic fibroblast growth factor (FGF; Sigma, USA), 10 mg/liter sodium heparin (Serva, Germany), 4 mM glutamine, 12.5 μM β-mercaptoethanol, 5 mg/liter iron-saturated human transferrin (Behring Werke AG, Germany), 2.1 g/liter $NaHCO_3$, 65 mg/liter benzyl-penicillin, and 100 mg/liter streptomycin sulfate [11]. HL-60 cells were used for the adhesion test. HL-60 cells were maintained in DMEM basal medium supplemented with 5% fetal calf serum.

Adhesion assay. HUVECs were seeded at the density of $1.9 \cdot 10^4$ cells/cm^2 in fibronectin/collagen-coated (2.5 μg/ml fibronectin and 5 μg/ml collagen) single-well polystyrene chamber sides (Nunc Inc., USA) and cultivated until subconfluence. Cells were then incubated for 4 h with recombinant human (rh) IL-1β (8 ng/ml), (rh) TNF-α (8 ng/ml) (Intragen, USA), or LPS (10 μg/ml) (Sigma). Pre-washed suspension of human leukemia cell line (HL-60) cells was then added to HUVECs at a 10 : 1 ratio and the mixture was incubated for 30-60 min. After several gentle washings the cultures were fixed with 4% glutaraldehyde for 40 min, dehydrated by sequential ethanol treatment in increasing concentrations, dried, and mounted in Eukit (O Kindler GmbH, Germany). The adherent HL-60 cells were counted with an automated microscope counting system (Adhex Cell Screen System; Germany) on 14 fields (515 × 515 μm per slide).

Immunohistochemistry. HUVECs were seeded in 4-well polystyrene chamber sides (Nunc) and cultivated until subconfluence. Cells were then incubated for 4 h with IL-1β (8 ng/ml), or TNF-α (8 ng/ml), or LPS (10 μg/ml) and washed three times. The samples were then fixed by 7% formalin and washed three times. To block nonspecific antibody binding, cells were incubated in 3% bovine serum albumin (BSA) for 15 min and then incubated for 30 min with the primary antibody (polyclonal goat antiserum specific for human E-selectin; Dianova, Germany; dilution 1 : 50), washed three times, and incubated with the secondary antibody coupled with dichlorotriazinylamino fluorescein (DTAF) (mouse-anti-goat antibody, diluted 1 : 50 in saturation buffer). After three washes, cells were incubated for 15 min in 10^{-5}% (w/v) 4,6-diamine-2-phenylindole-dihydrochloride (DAPI, Boehringer, Germany) in PBS to visualize cell nuclei. After the final wash, slides were dried and mounted in 20% (w/v) Mowiol (Hoechst, Germany). As negative control, parallel samples were stained with the secondary DTAF-labeled antibody only. A Zeiss Axiovert (Germany) microscope with epi-illumination for fluorescence was used for microphotography.

For immunohistochemical localization of $GbOse_3Cer$ and $GbOse_4Cer$, subconfluent layer of HUVECs was cultured in a serum-free medium supplemented with 0.01% BSA for 24 h to enter a quiescent state. Separate cell samples were then incubated with recombinant human TNF-α in increasing doses the for 4 h, washed, fixed as described before, and then incubated for 1 h with anti $GbOse_4Cer$ or $GbOse_3Cer$ chicken antisera in the dilution 1 : 40 [11]. The antibodies were the same as those used for immunooverlay TLC. Control cultures were incubated with pre-immune chicken antibody at the same dilution. After three washes, cells were incubated for 1 h with DTAF-labeled secondary antibodies directed against chicken IgY and diluted 1 : 40 in 3% BSA/PBS. After three washes, cells were incubated with DAPI, washed, dried, and embedded in Mowiol. Parallel control cultures were stained only with the secondary DTAF-labeled antibody. Antibody staining was counted morphometrically as ratio of area stained by antibody and area of nuclei stained by DAPI. Morphometry was performed using Autocad software (Autodesk GmbH, Germany).

Statistical analysis. Data are given as means ± standard deviations. Multiple group comparison was performed using ANOVA and HSD post-hoc test (SPSS software, version 11.5; SPSS Inc, USA).

RESULTS

E-Selectin expression in primary HUVECs cultures was assessed after stimulation with IL-1β, TNF-α, or

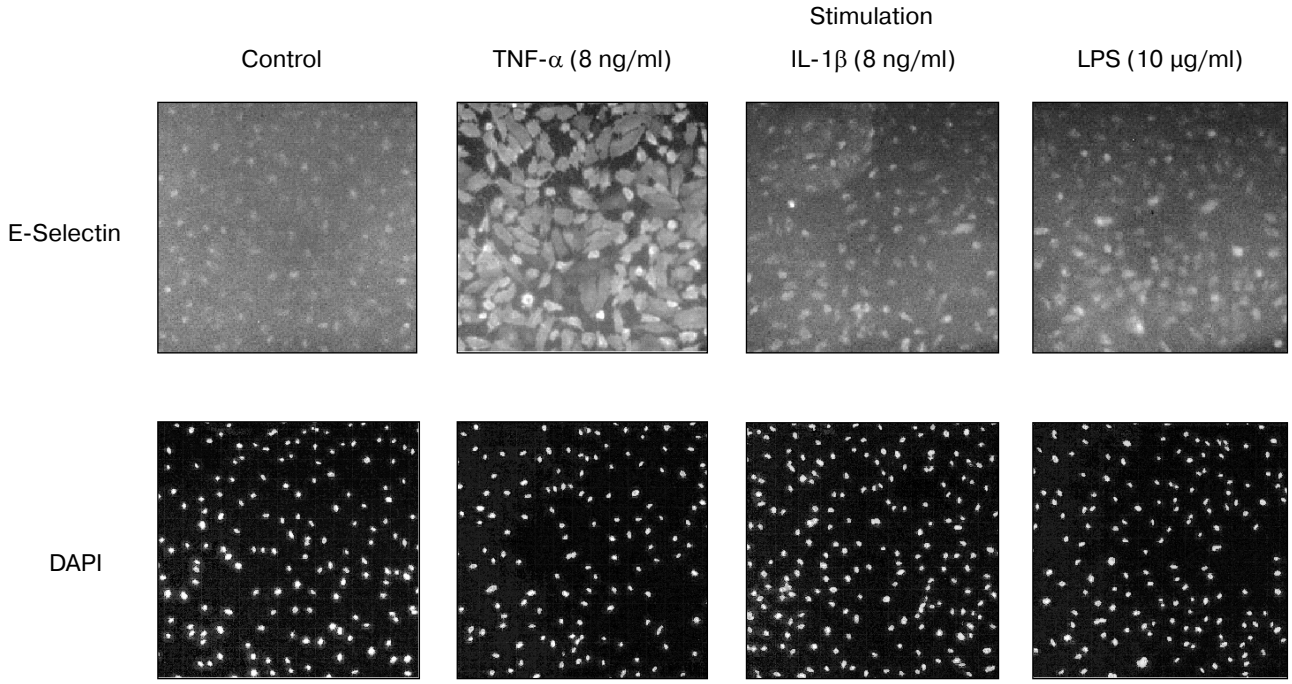

Fig. 1. Expression of E-selectin on HUVEC cells. Quiescent cells were stimulated with TNF-α, IL-1β, or LPS for 4 h, fixed, and stained with E-selectin-specific antibody.

LPS (Fig. 1). Resting cells did not express E-selectin, while stimulated cells did. TNF-α was the most potent stimulator of E-selectin expression, whereas the cell stimulation with IL-1β and LPS was less efficient.

To test the functional activation of HUVECs with cytokines, we used the ability of HL-60 cells to bind to endothelial cells. HUVECs stimulated with TNF-α, the most potent activator of E-selectin expression, bound HL-60 cells most efficiently compared to IL-1β or LPS, less efficient activators of E-selectin expression (Fig. 2). Automated assessment of HL-60 attachment to HUVECs confirmed the difference between these stimulating agents, the number of adherent HL-60 cells per field without stimulation being 3.43 ± 0.26 while 353.12 ± 31.62, 3.48 ± 0.33, and 7.92 ± 0.35 with stimulation either with TNF-α, IL-1β, or LPS, respectively. The difference revealed by the ANOVA test was shown to be statistically significant for TNF-α stimulation compared to all other groups ($p < 0.001$). TNF-α stimulated E-selectin expression in a dose-dependent manner (Fig. 3a).

Parallel cultures of HUVECs were stained with $GbOse_3Cer$ or $GbOse_4Cer$ antibodies (Fig. 3). Quiescent cells expressed abundant $GbOse_3Cer$ or $GbOse_4Cer$. Stimulation with TNF-α significantly increased the overall intensity of the GSLs staining in a dose-dependent manner (table) and affected GSL localization and distribution (Fig. 3, b and c). $GbOse_3Cer$ and $GbOse_4Cer$ antibodies stained both cell membrane and cytoplasmic

Effect of TNF-α on $GbOse_3Cer$- and $GbOse_4Cer$-directed staining in HUVEC cells

Globoside	Relative intracellular staining			
	control	TNF-α (0.5 ng/ml)	TNF-α (2.5 ng/ml)	TNF-α (10 ng/ml)
$GbOse_3Cer$	1.53 ± 0.48	2.36 ± 0.80*	2.32 ± 0.56*	2.25 ± 0.44*
$GbOse_4Cer$	1.20 ± 0.28	2.25 ± 0.91*	1.95 ± 0.61*	1.71 ± 0.80

Note: Intracellular staining was expressed as the area of antibody staining relative to the area of cell nucleus. Antibody staining area was measured on DTAF-stained cells as in Fig. 3 (b and c), and nucleus area on the same cells stained with DAPI was measured also. The absolute nucleus area did not significantly differ between groups. The results are expressed as means of measurements with 12-18 cells. Asterisks indicate statistically significant difference vs. control cells ($p \leq 0.05$, ANOVA and HSD post-hoc test).

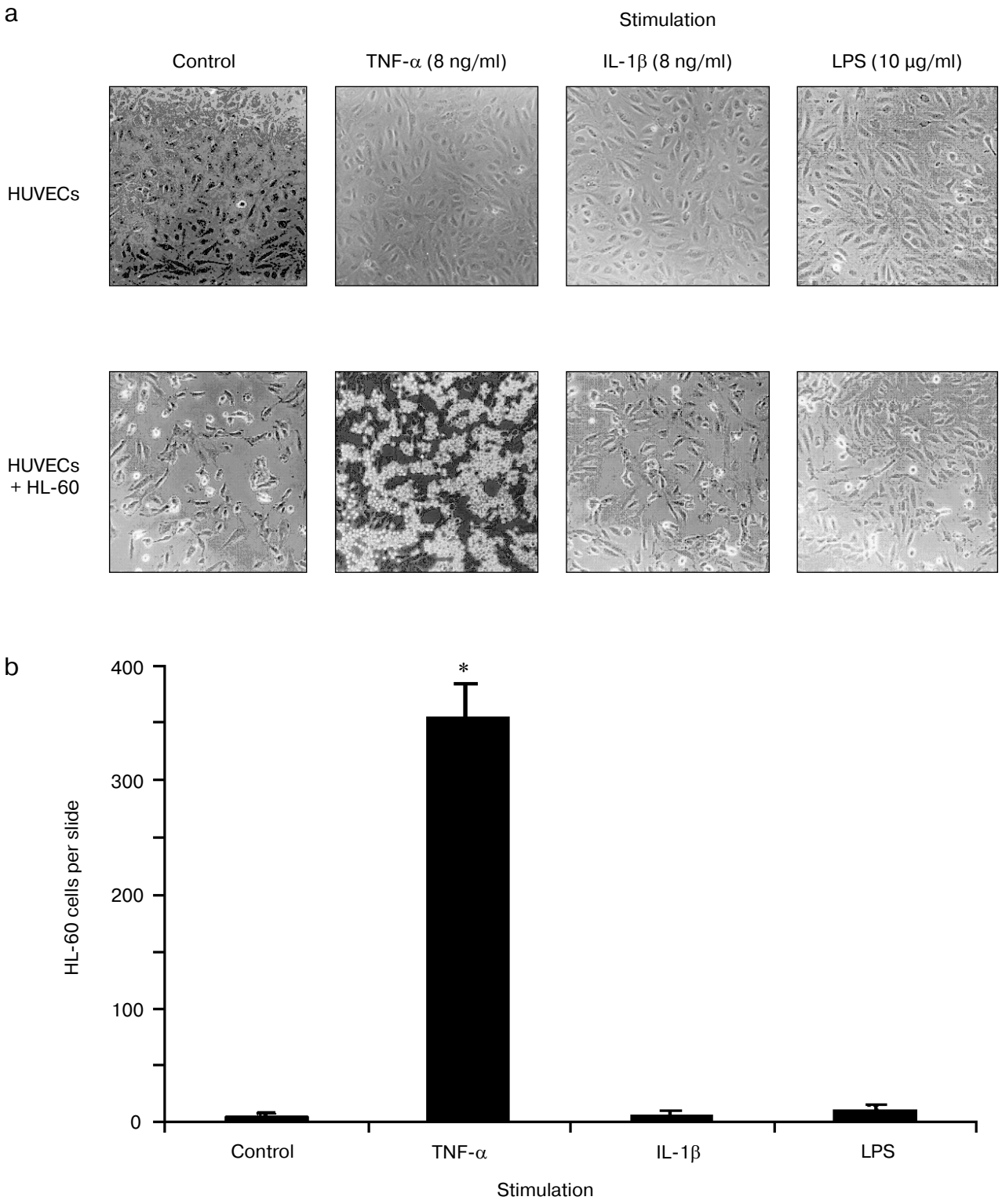

Fig. 2. a) Adhesion of HL-60 cells to HUVEC cells. Quiescent HUVEC cells were stimulated with TNF-α, IL-1β, or LPS for 4 h and processed for adhesion test with HL-60 cells. b) Effect of TNF-α, IL-1β, or LPS on the adhesion of HL-60 cells to HUVECs. Number of adherent HL-60 cells to HUVEC cells per slide (mean ± SD) with or without stimulation with TNF-α (8 ng/ml), IL-1β (8 ng/ml), or LPS (10 μg/ml) are given. Asterisk indicates statistically significant difference vs. other groups ($p < 0.001$, ANOVA and HSD post-hoc test).

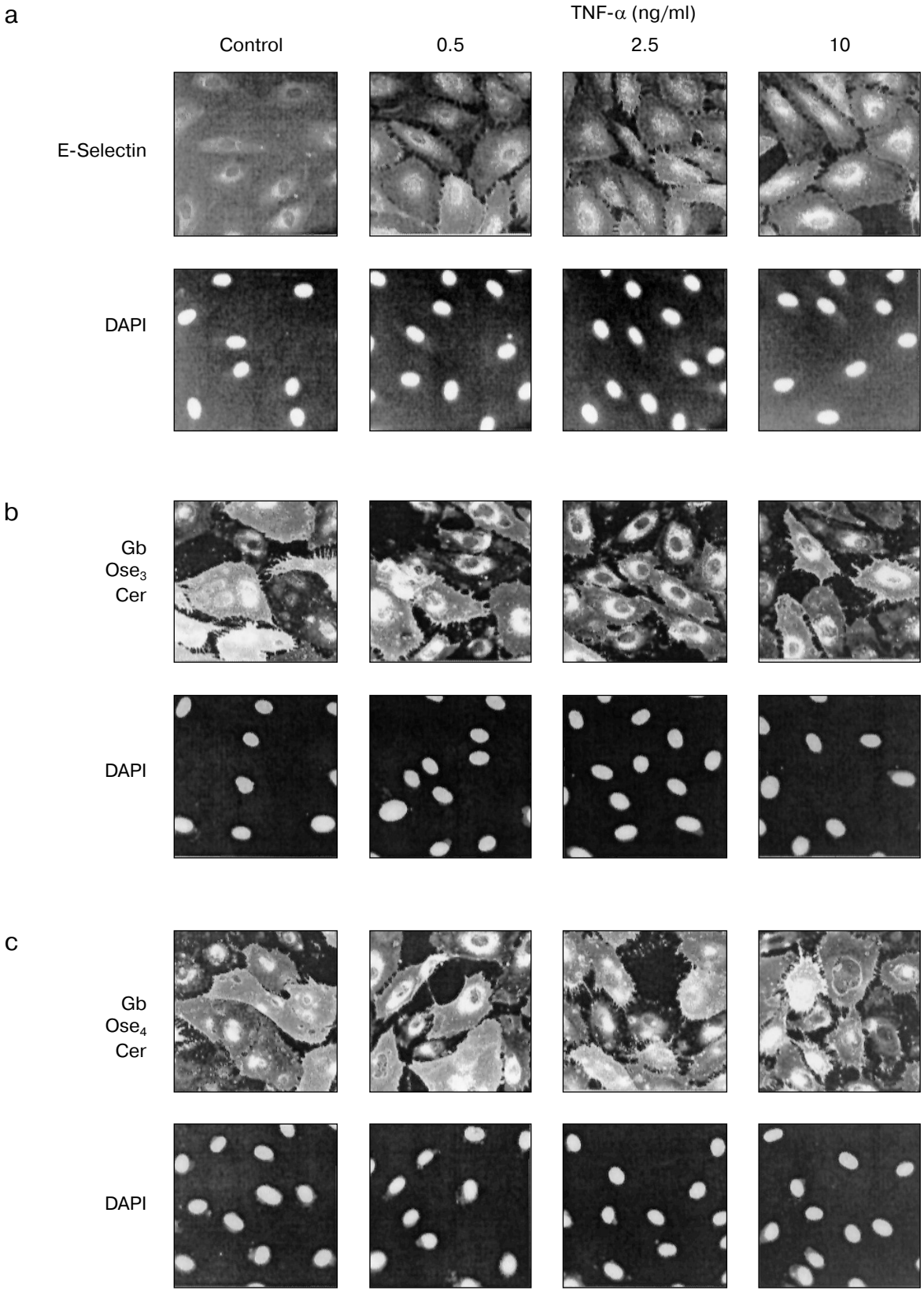

Fig. 3. Effect of TNF-α on the expression of E-selectin (a), $GbOse_3Cer$ (b), and $GbOse_4Cer$ (c) in HUVEC cells. Quiescent cells were stimulated with increasing doses of TNF-α for 4 h.

structures in non-stimulated cells, the staining pattern being changed in stimulated cells, especially for $GbOse_3Cer$.

DISCUSSION

Our study showed that cytokine-stimulated and functionally activated human endothelial cells changed the expression of neutral glycosphingolipid in parallel with that of E-selectin. This study also confirmed immunohistochemically that TNF-α was the most potent stimulator of E-selectin on HUVEC, whereas LPS and IL-1β were less effective [12]. This difference can be explained by different targets of the cytokines, since recent study showed that the central target for lipopolysaccharide action in HUVECs was Rho/Rho kinase rather then E-selectin [13]. In addition, lipopolysaccharide induced interleukin-1 receptor antagonist type I mRNA in HUVECs [14].

Neutral glycosphingolipids $GbOse_3Cer$ and $GbOse_4Cer$ that have been described as receptors for *Escherichia coli* in pigs and humans [15, 16] may play an important role in immunological response [17]. Xia et al. [18] showed recently that TNF-α simultaneously and independently activated two antagonistic biochemical signaling pathways in HUVECs, sphingomyelinase and sphingosine kinase pathways, the balance of which could regulate the fate of a cell in response to TNF-α stimulation. Bioactive sphingolipid metabolites have diverse effects, and the particular function of a cytokine may be explained by the selectivity for the enzymes in question. TNF-α has a potential role in the activation of enzyme UDP-galactose-glucosylceramide β(1→4) galactosyltransferase (GalT-2), which generates lactosylceramide (LacCer). TNF-α can increase LacCer expression which consequently stimulates ICAM-1 both at transcriptional and translation levels, this facilitating the adhesion of polymorphonuclear leukocytes (PMNs) to endothelial cells [19].

As sphingolipid-mediated biology is a matter of balance between currently available precursors and the enzymatic machinery inside the cell, the elongation products of LacCer, $GbOse_3Cer$, and $GbOse_4Cer$ generated in the Golgi by the action of corresponding transferases could also be involved in such reactions to TNF signal transduction [20]. Stricklett et al. showed recently that TNF and other inflammatory cytokines up-regulated, most likely via transcription, activities of three enzymes involved in $GbOse_3Cer$ synthesis in human brain endothelial cells [21].

Our results show that distribution of $GbOse_3Cer$ and $GbOse_4Cer$ in HUVECs was TNF-α-dependent. Small dose of TNF-α caused predominant staining around the nucleus, intensely stained cytoplasmic patches close to the cell membrane were induced by higher doses and their integrity with membrane somewhere could be seen under the highest dose applied. Similar redistribution of $GbOse_3Cer$ was described after γ-interferon treatment [9]. γ-Interferon affected redistribution of $GbOse_3Cer$ between surface and intracellular compartments of HUVECs [9]. Golgi seems to be the central point in lipid metabolism as recently proposed by Baron and Malhotra [22]. These authors have suggested an intimate connection between lipid metabolic pathways and Golgi activity [22]. Fukunaga et al. [23] demonstrated recently that long chain ceramides enhanced brefeldin A-induced Golgi disassembly and also tubulation of the *trans*-Golgi network, endosomes, and lysosomes. Ceramide is a structural component of $GbOse_4Cer$ and its level is increased by the action of TNF-α [24]. Hence, redistribution of $GbOse_3Cer$ observed in our study could be caused by the abundance of ceramide, primarily induced by TNF-α. Two recent studies showed that TNF-α stimulated expression of $GbOse_3Cer$ in brain endothelial cells. TNF-α increased $GbOse_3Cer$ content in human cerebral endothelial cells and sensitivity to Shiga toxin [25]. Shiga toxin, also referred as verotoxin 1, is an *Escherichia coli*-derived ligand of $GbOse_3Cer$, which activates a sphingomyelin/ceramide pathway [25]. Human brain microvascular endothelial cells produced only trace amounts of TNF-α when stimulated with purified Shiga toxin *in vitro*, but the treatment with TNF-α was associated with the increased expression of $GbOse_3Cer$ [26]. In contrast, verotoxin 1 can directly affect $GbOse_3Cer$ expression in bovine lymphocytes, rather than inducing a cytokine-mediated effect [27].

In conclusion, our results represent the first evidence of the effect of TNF-α on $GbOse_3Cer$ and $GbOse_4Cer$ expression in HUVECs. Further experiments that could apply monoclonal antibodies against globo-series GSL, inhibitors of TNF-α receptor, or transfected cells without TNF-α receptor are needed to reveal the possible role of $GbOse_3Cer$ and $GbOse_4Cer$ in immunological response of endothelial cells.

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