Lactosylceramide Mediates Tumor Necrosis Factor-α-induced Intercellular Adhesion Molecule-1 (ICAM-1) Expression and the Adhesion of Neutrophil in Human Umbilical Vein Endothelial Cells*

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The endothelial expression of adhesion molecules by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) has been suggested to contribute to the initiation of atherosclerotic plaque formation. Since lactosylceramide (LacCer) accumulates in large quantities in human atherosclerotic plaque, we have explored its role in TNF-α-induced expression of intercellular adhesion molecule-1 (ICAM-1) in human umbilical vein endothelial cells and their consequent adhesion to polymorphonuclear leukocytes (PMNs). We found that TNF-α increased LacCer synthesis by way of stimulating the activity of UDP-galactose:glucosylceramide β(1→4)-galactosyltransferase in a time-dependent fashion. The TNF-α-induced expression of ICAM-1 was abrogated by D-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), an inhibitor of UDP-galactose:glucosylceramide β(1→4)-galactosyltransferase. However, the addition of LacCer reversed the D-PDMP effect on TNF-α-induced ICAM-1 expression in human umbilical vein endothelial cells. Northern hybridization analysis of mRNA levels and enzyme-linked immunosorbent assays revealed that LacCer (5 μM) specifically stimulated ICAM-1 at both the transcriptional and translational levels. This was accompanied by the adhesion of PMNs, which was visualized by confocal microscopy. Further studies revealed that LacCer stimulated the endogenous generation of superoxide radicals (O2•−) about 5-fold compared with the control by specifically activating plasma membrane-associated NADPH-dependent oxidase. This phenomenon was blocked by the antioxidant N-acetyl-l-cysteine, pyrrolidine dithiocarbamate, and the NADPH oxidase inhibitor, diphenylene iodonium. Overexpression of endogenous CuZn-superoxide dismutase via an adenoviral vector carrying cDNA for CuZn-superoxide dismutase, also inhibited LacCer-induced ICAM-1 expression in endothelial cells. In sum, our findings suggest that LacCer may play the role of a lipid second messenger in TNF-α-induced pathogenesis by activating an oxidant-sensitive transcriptional pathway that leads to the adhesion of PMNs to endothelial cells.

One of the critical events in the pathogenesis of atherosclerotic lesion formation is the focal accumulation of lipid-laden foam cells beneath an intact arterial endothelial lining (1, 2). Localized attachment of circulating monocytes and lymphocytes to the arterial endothelium appears to precede the formation of early foam cell lesions (1, 2). Various studies have demonstrated that the intercellular cell adhesion molecule-1 (ICAM-1),1 E-selectin (endothelial leukocyte adhesion molecule; ELAM-1), and vascular cell adhesion molecule (VCAM-1) are all inducible on the endothelial surface following stimulation with TNF-α (3–5). ELAM-1 serves as a ligand for neutrophils (5), eosinophils (6), monocytes (7), and subpopulation of circulating lymphocytes (8). VCAM-1, which is induced on endothelial cells by IL-1, TNF-α, or IL-4 (9) serves as a ligand for very late antigen-4 on monocytes, lymphocytes, and eosinophils (10). ICAM-1 is a specific ligand for lymphocyte function-associated antigen-1 (11) and Mac-1 (CD11/CD18), which is expressed on neutrophils and monocytes (12). In vivo studies have suggested that both ICAM-1 and VCAM-1, which are inducible by TNF-α, are expressed in human atherosclerotic lesions (13). It is well documented that TNF-α induces the expression of cell adhesion molecules by activating an oxidant-sensitive signal transduction pathway (14, 15). However, to the best of our knowledge, the role of glycosphingolipids (GSLs), particularly lactosylceramide (LacCer), in this phenomenon has not been explored.

LacCer, a ubiquitous GSL, plays a pivotal role in the biosynthesis of complex glycosphingolipids (16). Moreover, the level of this GSL is elevated in several proliferative diseases including human polycystic kidney disease (17), familial hypercholesterolemia (18, 19), and atherosclerosis (20, 21). In addition, recently, there has been a surge in reports suggesting that sphingolipids may be implicated as second messengers in mediating diverse molecular events, including cell proliferation (22–24) and programmed cell death (apoptosis) (25), and as adhesive molecules (16). In this paper, we demonstrate that TNF-α stimulates the synthesis of LacCer by stimulating the activity of a UDP-galactose:glucosylceramide β(1→4)-galactosyltransferase (GalT-2). In turn, LacCer, via the generation of superoxide, up-regulates the expression of ICAM-1 on the surface of

1 The abbreviations used are: ICAM-1, intercellular cell adhesion molecule-1; GSL, glycosphingolipid; LacCer, lactosylceramide; GlcCer, glucosylceramide; Cer, ceramide; HUVEC, human umbilical vein endothelial cell; O2•−, superoxide; SOD, superoxide dismutase; VCAM-1, vascular cell adhesion molecule-1; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; NAC, N-acetyl-l-cysteine; DPI, diphenylene iodonium; PMN, polymorphonuclear leukocyte; MOLT, multiplicity of infection; D-PDMP, D-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; ELAM-1, endothelial leukocyte adhesion molecule-1; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; IL, interleukin; GaIT-2, UDP-galactose:glucosylceramide β(1→4)-galactosyltransferase; MOPS, 4-morpholinepropanesulfonic acid.
human umbilical vein endothelial cells (HUVECs) that facilitate the adhesion of neutrophils.

MATERIALS AND METHODS

Isotopes and Chemicals—[α-32P]dCTP (3000 Ci/mmol) and [α-32P]dATP (3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. UDP-[14C]galactose (2.04 GBq/mmol) and [3H]galactose (60 Ci/mmol) were purchased from NEN Life Science Products and American Radiocative Chemical Company (St. Louis, MO), respectively. Glycosphingolipids and all other chemicals were purchased from Sigma. The purity of glycosphingolipids was assessed by high pressure liquid chromatography and/or high performance thin layer chromatography to be >99%. Anti-ICAM-1 and anti-VCAM-1 antibodies were obtained from Pharmingen (San Diego, CA). Cell Tracker dye green and Cell Tracker dye orange were obtained from Molecular Probes, Inc. (Eugene, OR). Diphenylene iodonium (DPI) was from Calbiochem. SOD virus carrying cDNA for superoxide dismutase and containing 1% bovine serum albumin and 0.1 M glycine overnight at 4 °C.

Next, the medium was removed, and the monolayer was washed with sterile PBS to remove nonspecifically bound radioactivity. Fresh serum-containing 1% bovine serum albumin and 0.1 M glycine overnight at 4 °C. Eahy 926 cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml).

Carcin—HUVECs were purchased from Cloneteks. Eahy 926 cells, a hybridoma permanent endothelial cell line, derived from HUVECs and human epithelial cell line A549 (26), was a generous gift from Dr. Roger Harrison (University of Bath, United Kingdom). HUVECs were maintained in endothelial cell growth medium supplemented with bovine brain extract (27). Eahy 926 cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml).

Vehicle for Glycosphingolipids—Stock solutions of LacCer and other glycosphingolipids were prepared in Me2SO as described (23) and added to culture medium to achieve the desired concentrations of LacCer. The final MeSO concentration exposed to cells was 0.01%. DI water stock solutions were also prepared in MeSO and stored at −20 °C until use. Cells incubated with 0.01% MeSO served as a control. An aqueous solution of N-acetylgalactosamine was prepared in the culture medium.

Cell Surface ICAM-1, VCAM-1, and E-selectin Expression Assay—The quantitative expression of ICAM-1, VCAM-1, and E-selectin on the surface of the endothelial cell monolayers was determined by a modified ELISA in 96-well plates (28). Following incubation with antagonists and agonists, endothelial cell monolayers were fixed with 3.7% formaldehyde (pH 7.4) containing 0.1% 3-lysine monohydrochloride and 0.01% sodium azide for 20 min at 4 °C and then blocked with PBS containing 1% bovine serum albumin and 0.1% glycine overnight at 4 °C. The fixed monolayer was then incubated with mouse monoclonal anti-human ICAM-1 or anti-human VCAM-1 or anti-human E-selectin for 1 h at 37 °C. Next it was incubated with peroxidase-conjugated anti-mouse IgG (Fab')2, for 1 h at 37 °C. After washing, a developing substrate (0.4 mg/ml O-phenylenediamine in 0.2 M H2SO4; and 1 mM H2O2, pH 7.0) was added to each well, and radioactivity was stopped with 2 M H2SO4. The plates were read on a spectrophotometric plate reader at 520 nm (28). ICAM-1 expression was assessed qualitatively in Eahy 926 cells by immunofluorescence staining using fluorescein isothiocyanate (FITC)-conjugated IgG following fixing of cells incubated with or without LacCer with 3% formaldehyde as described above.

Measurement of GalT-2 Activity—The activity of GalT-2 in cells incubated with TNF-α was measured employing UDP-[14C]galactose as a nucleotide sugar donor and glucosylceramide as an acceptor as described previously (29). Briefly, the GalT-2 assay mixture contained 100 μg of enzyme preparation, 20 μM diphenylene iodonium (DPI), 0.01% Me2SO served as a control. An aqueous solution of N-acetylgalactosamine was prepared in the culture medium.

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Measurement of LacCer Synthesis— Cultures of HUVECs were incubated in growth medium containing [3H]galactose (5 μCi/ml) for 24 h. Next, the medium was removed, and the monolayer was washed with sterile PBS to remove nonspecifically bound radioactivity. Fresh serum-free medium (5 ml/pate) was added. One set of dishes was incubated with p-PDMP (20 μM) in PBS for 2 h. Next, TNF-α (300 units/ml) was added to cells incubated with or without p-PDMP. At various time points, cells were harvested and washed with PBS and centrifuged. The cell pellet was subjected to extraction of lipids with organic solvents, and glycosphingolipids were purified as described under “Materials and Methods.” The radiolabeled glycosphingolipids were separated by high performance thin layer chromatography using chloroform/methanol/water (100:42:6, v/v/v) as the developing solvent. The chromatographic plate was dried in air and stained with iodine vapor. The gel area corresponding to LacCer was scraped, and radioactivity was measured employing “liquiscint” (NEN Life Science Products) as a scintillating fluid.

Northern Blot Analysis of ICAM-1 mRNA Expression in HUVECs—Total cellular RNA was isolated (30) from HUVECs that were preincubated with 15 mM NAC for 30 min, 5 μM diphenylene iodonium for 30 min, followed by stimulation with the indicated concentrations of LacCer/TNF-α for 2 h. Twenty micrograms of total RNA were separated by electrophoresis on 1% agarose gel (25 mM MOPS, pH 7.8, 1 mM EDTA, 1% (v/v) formaldeyde), transferred to a nylon membrane blotting membrane (Bio-Rad) and hybridized with a 32P-labeled ICAM-1 cDNA probe as described. The transfer of RNA to membrane and equal amount RNA loading was confirmed by ethidium bromide staining.

Measurement of Superoxide Generation in Intact HUVECs—Lucigenin, an acridylium compound that emits light upon interaction with O2−, was used to measure O2 production by chemiluminescence (23). Confluent grown endothelial cells were harvested, and O2− generation from the intact endothelial cell suspension was measured using a 96 well plate containing dark-adapted lucigenin (250 μM) in balanced salt solution, as described by Bhunia et al. (23). The viability of the suspended cells was >90% as determined by trypan blue exclusion. LacCer or other GSL solutions were added as putative agonists, and the resultant increases in photon emission were measured every 20 s thereafter for 10 min in a scintillation counter (Packard TOP counter). The net increase in O2− generation at each time point was calculated by compar-
following modified Wright-Giemsa staining. The PMNs were labeled by a single-step protocol for 2 h followed by stimulation with TNF-α for 12 h. Next, cells were fixed with 3.7% formaldehyde in PBS and ICAM-1 expression was measured by modified ELISA using monoclonal ICAM-1 antibody as described under "Materials and Methods." Cells incubated with 0.01% Me2SO served as a control. In a parallel experiment, following stimulation with TNF-α (100 units/ml), D-PDMP decreased the synthesis of LacCer 0.4-fold compared with the control. D-PDMP effect was not altered significantly among the three TNF-α groups.

RESULTS

TNFα Stimulates LacCer Synthesis in a Time- and Concentration-dependent Fashion in HUVECs—We found that TNF-α (both 100 and 300 units/ml) stimulated GaIT-2 activity in a time-dependent manner (Fig. 1A). Within 5 min, a 1.5-fold stimulation of GaIT-2 activity was observed compared with the control. Thereafter, the activity of GaIT-2 continued to rise in cells stimulated with TNF-α. This phenomenon was accompanied by a time-dependent increase in the synthesis of LacCer as evidenced by an increase in the incorporation of [3H]galactose into LacCer in cells stimulated with TNF-α (Fig. 1B). For example, 5 min after the stimulation with TNF-α, a 1.5-fold increase in LacCer synthesis was observed and continued to increase 60 min after stimulation. Preincubation of cells with D-PDMP decreased the synthesis of LacCer 0.4-fold compared with the control. D-PDMP effect was not altered significantly after stimulation of cells with TNF-α.

FIG. 2. Effects of d-PDMP on TNF-α- and LacCer-mediated expression of ICAM-1 in HUVECs. A, d-PDMP inhibits TNF-α-induced ICAM-1 expression. Cells were preincubated with various concentrations of d-PDMP for 2 h followed by stimulation with TNF-α for 12 h. Next, cells were fixed with 3.7% formaldehyde in PBS, and ICAM-1 expression was measured by modified ELISA using monoclonal ICAM-1 antibody as described under "Materials and Methods." Cells incubated with 0.01% Me2SO served as a control. In a parallel experiment, following stimulation with TNF-α (100 units/ml), D-PDMP decreased the synthesis of LacCer 0.4-fold compared with the control. D-PDMP effect was not altered significantly among the three TNF-α groups.

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FIG. 3. Effects of LacCer and its homologs on cell adhesion protein expression in HUVECs. A, confluent cultures of HUVECs grown in a 96-well plate (1 × 10^4 cells/well) were incubated with various glycosphingolipids (5 μM) for 8 h. Next, cells were fixed with 3.7% formaldehyde in PBS, and ICAM-1 expression was measured by a modified ELISA assay using monoclonal ICAM-1 antibody as described under “Materials and Methods.” Cells incubated with 0.01% Me_2SO served as a control. B, effect of time of incubation with TNF-α (100 units/ml) and LacCer (5 μM) on ICAM-1 expression. Cells incubated with 0.01% Me_2SO served as a control. C, effects of LacCer on ICAM-1 expression in endothelial cells. ICAM-1 expression was measured following the stimulation of cells with various concentrations of LacCer for 8 h, as described earlier. E, control; LacCer; TNF-α (100 units/ml).
LacCer Mediates TNF-\(\alpha\)-Signaling

**Fig. 4.** Northern blot analysis of ICAM-1 mRNA expression following incubation with LacCer and TNF-\(\alpha\) in HUVECs. ICAM-1 mRNA expression was measured following preincubation of cells with 15 mM NAC or 5 \(\mu\)M DPI for 30 min followed by incubation with 5 \(\mu\)M LacCer or 300 units/ml TNF-\(\alpha\) for 2 h as described under “Materials and Methods.” Images of the ethidium bromide (EtBr)-stained \(\zeta\)-probe blotting membrane were taken to demonstrate equal amount of RNA loading and transfer. Two such experiments were conducted, and the results of a representative experiment are shown.

ICAM-1 Expression Is Abrogated by \(\delta\)-PDMP and Bypassed by LacCer but Not by GlcCer—Preincubation of HUVECs with \(\delta\)-PDMP inhibited TNF-\(\alpha\)-induced ICAM-1 expression in a concentration-dependent manner (Fig. 2A). Moreover, this inhibition by \(\delta\)-PDMP was reversed upon incubation with exogenous LacCer but not by exogenous ceramide or glucosylceramide (Fig. 2B), suggesting the involvement of LacCer in TNF-\(\alpha\)-induced ICAM-1 expression.

LacCer Stimulates the Expression of ICAM-1 in Endothelial Cells—HUVECs were incubated with various GSLs (5 mM) and their constituents. Only LacCer stimulated the expression of ICAM-1. Other GSLs and their constituents or gangliosides did not stimulate ICAM-1 expression (Fig. 3A). LacCer stimulated ICAM-1 expression in a time-dependent (Fig. 3B) and concentration-dependent (Fig. 3C) manner. Maximal stimulation of ICAM-1 (9-fold compared with control) was observed with 5 \(\mu\)M LacCer at 8 h (Fig. 3, B and C). LacCer (5 \(\mu\)M) and TNF-\(\alpha\) (100 units/ml) both exerted a time-dependent stimulation of ICAM-1 (Fig. 3B). However, unlike LacCer, TNF-\(\alpha\) also stimulated the expression of VCAM-1 and E-selectin in addition to ICAM-1 (Fig. 3, D and E). Moreover, maximum stimulation of ICAM-1 (10-fold), VCAM-1 (9-fold), and E-selectin (10-fold) was observed after 12 h of incubation with TNF-\(\alpha\). Immunofluorescence visualization, using ICAM-1 antibody followed by incubation with FITC-conjugated anti-mouse IgG, further confirmed that LacCer induced the stimulation of cell surface ICAM-1 expression in intact Eahy 926 endothelial cells as compared with control (Fig. 3F).

LacCer Stimulates the Expression of ICAM-1 mRNA Levels in HUVECs—As shown in Fig. 4, LacCer exerted a concentration-dependent increase in the mRNA levels of ICAM-1 in HUVECs. A maximum stimulation of ICAM-1 mRNA level occurred following incubation of cells with 5 \(\mu\)M LacCer for 2 h. Moreover, this phenomenon was abrogated by preincubation of cells with \(N\)-acetyl-L-cysteine, an antioxidant and a scavenger of free oxygen radicals, as well as DPI, an inhibitor of NADPH oxidase. Interestingly, the magnitude of stimulation of ICAM-1 gene transcription by LacCer was comparable with TNF-\(\alpha\).

LacCer Induces the Generation of Superoxide in Endothelial Cells—Since TNF-\(\alpha\) induces ICAM-1 expression via redox signaling, i.e. generation of reactive oxygen species, we examined whether LacCer also generates \(O_2^-\) for the induction of ICAM-1 in endothelial cells. Direct measurement of \(O_2^-\) by lucigenin chemiluminescence revealed that LacCer stimulated \(O_2^-\) production in HUVECs in a concentration-dependent (Fig. 5A) and time-dependent (Fig. 5B) manner. Maximum stimulation in the generation of \(O_2^-\) (5-fold), compared with the control, was observed following the incubation of cells with 5 \(\mu\)M LacCer for 5 min.

LacCer Specifically Stimulates NADPH Oxidase-dependent \(O_2^-\) Generation in HUVECs—At various time points following stimulation with or without (5 \(\mu\)M) LacCer, NADPH oxidase activity was measured in the membrane preparations (Fig. 6). Three-fold stimulation of NADPH oxidase activity was observed at 5 \(\mu\)M LacCer, as compared with nonstimulated cell membrane preparations (Fig. 6A). Preincubation of LacCer-stimulated/nonstimulated cell membrane preparations with DPI, a potent NADPH oxidase inhibitor (23), attenuated the LacCer induced increase in NADPH-dependent oxidase activity in the membrane preparations (Fig. 6A). In contrast, LacCer did not alter the activity of NADPH oxidase activity in stimulated/nonstimulated membrane preparations (Fig. 6B). Neither NADPH oxidase nor NADPH oxidase activity was observed in the LacCer-stimulated/unstimulated cell cytosol (data not shown). On the other hand, allopurinol, a specific inhibitor of xanthine oxidase (23), failed to inhibit LacCer-induced \(O_2^-\) generation (data not shown). Since rotenone also failed to inhibit NADPH oxidase activity in the membrane fraction, it appears that plasma membrane-associated NADPH oxidase is the source of LacCer-induced \(O_2^-\) generation in endothelial cells.

NAC and DPI Abrogate LacCer-induced Superoxide Generation and ICAM-1 Expression—Preincubation of cells with 5 \(\mu\)M DPI for 30 min failed to respond to the LacCer-mediated increased generation of \(O_2^-\) (Fig. 7A) and ICAM-1 expression (Fig. 7B). Similarly, preincubation of cells with \(N\)-acetyl-L-cysteine (15 mM) for 30 min also abrogated LacCer-induced generation of \(O_2^-\) as well as ICAM-1 expression (Fig. 7, A and B). However, exogenously added SOD did not inhibit the LacCer-induced \(O_2^-\) levels in endothelial cells and ICAM-1 expression (Fig. 7, A and B). These findings suggest that the NADPH oxidase-dependent endogenous generation of \(O_2^-\) is necessary to mediate LacCer-induced ICAM-1 expression.

Overexpression of Intracellular SOD Inhibits LacCer-induced ICAM-1 Expression—Since exogenous SOD did not inhibit LacCer-induced \(O_2^-\) levels and ICAM-1 expression, we overexpressed SOD via adenovirus-mediated gene transfer. The expression of SOD in Ad-SOD-infected endothelial cells was 2.5-fold higher at 50 MOI compared with that of Ad-\(\beta\)-galactosidase-infected cells, as measured by immunofluorescence using monoclonal anti-SOD (Fig. 8A). The increase in SOD activity in the Ad-SOD-infected endothelial cell lysate was paralleled with an increase in MOI (Fig. 8B). SOD enzymatic activity in Ad-SOD-infected endothelial cells was also increased, dose-dependently, compared with that of the control (Ad-\(\beta\)-galactosidase-infected) cells (Fig. 8B). In the Ad-\(\beta\)-galactosidase-infected control cells, LacCer stimulated ICAM-1 expression, as shown in Fig. 4.

- LacCer. D, effects of TNF-\(\alpha\) and LacCer on the expression of VCAM-1 in HUVECs. Following incubation with LacCer (5 \(\mu\)M) or TNF-\(\alpha\) (300 units/ml), cells were fixed, and VCAM-1 expression was measured by ELISA using anti-human VCAM-1 antibody. E, effects of TNF-\(\alpha\) and LacCer on the expression of E-selectin in HUVECs. The protocol in this experiment was similar to that described for D except that the level of E-selectin expression was measured employing anti-human E-selectin antibody. Each point is the mean ± S.D. of five separate experiments. F, immunofluorescence assay of ICAM-1. Eahy 926 cells (endothelial cells) were confluent grown in glass chamber slides and incubated with or without 5 \(\mu\)M LacCer for 8 h. Next, cells were fixed in 3.7% formaldehyde, and ICAM-1 expression was monitored by immunofluorescence using ICAM-1 monoclonal antibody followed by incubation with FITC-conjugated anti-mouse IgG and photographed employing fluorescence microscopy. Data presented here are from one of the two separate experiments with identical results.
LacCer Mediates TNF-α Signaling

**DISCUSSION**

Significant information has been generated supporting the concept that TNF-α plays an important role in the adhesion of monocytes and neutrophils to the endothelial cells (3–5). This phenomenon precedes the production of superoxides, activation of NF-κB, and an increase in the expression of cell adhesion molecules such as ICAM-1 in endothelial cells (14, 15). This oxidant-sensitive transcriptional pathway plays an important role in vascular lesion formation (1, 2). In the present study, we have extended the mechanism over previous observations by demonstrating that TNF-α increases the synthesis of LacCer via activating GalT-2. In turn, LacCer induced ICAM-1 expression and PMN adhesion. This may be an early process in the TNF-α-mediated atherosclerotic lesion formation. Our current hypothesis describing how LacCer serves as a lipid second messenger in the activation of an oxidant-sensitive signal transduction pathway that leads to the adhesion of neutrophil to endothelial cells is summarized in Fig. 11.

LacCer is a ubiquitous glycosphingolipid and plays a pivotal role in the biosynthesis of complex glycosphingolipids (16). Moreover, LacCer may contribute to proliferative diseases such as polycystic kidney disease, atherosclerosis (17), and familial hypercholesterolemia (18, 19). The biological function of LacCer is further substantiated by its role in vascular lesion formation, as polycystic kidney disease, atherosclerosis (17), and familial hypercholesterolemia (18, 19).

**FIG. 5. Effects of LacCer concentration and time of incubation on the generation of superoxide in HUVECs.** Confluently grown endothelial cell monolayers were harvested and suspended in balanced salt solution. Next, the rate of generation of superoxide was measured by lucigenin chemiluminescence as described under “Materials and Methods.” A, effect of various concentrations of LacCer on the rate of generation of superoxide in endothelial cells. ○, control (vehicle 0.01% Me2SO); ●, LacCer. B, effect of time of incubation with LacCer (5 μM) on the rate of generation of superoxide. ○, control (vehicle, 0.01% Me2SO); ●, LacCer. Each point is the mean ± S.D. of five separate experiments.

**FIG. 6. Effects of LacCer on the activity of NADPH oxidase and NADH oxidase in HUVECs.** A, plasma membrane preparation of cells incubated with or without LacCer were used for the determination of NADPH oxidase activity (expressed as nmol/min/mg of protein) as described under “Materials and Methods.” Shown are NADPH oxidase activity in control cells (○) and 5 μM LacCer-treated cells (●) and NADPH oxidase activity (expressed as nmol/min/mg of protein) in cells incubated with 5 μM DPI for 30 min and in membranes from cells incubated with 5 μM DPI for 30 min plus 5 μM LacCer (●). B, NADH oxidase activity in membrane in control cells (○) and in cells incubated with LacCer (●). The activities of NADPH oxidase and NADH oxidase are expressed as nmol/min/mg of protein. Each point is the mean ± S.D. of four separate experiments.
cle cells, oxidized low density lipoprotein stimulated GalT-2 activity and LacCer production. LacCer in turn served as a surrogate for oxidized low density lipoprotein to induce cell proliferation (37, 38). In the present study with endothelial cells, a parallel observation was made in which TNF-α also activated GalT-2 and produced LacCer. Accordingly, we focused our studies on the upstream step in this signaling pathway. We employed D-PDMP as an inhibitor of glycosyltrans-
generation, since this method detects generation in HUVECs levels in hypercholesterolemia (45). Since the level of human neutrophils were preincubated with either 5 μM DPI or 15 mM NAC for 30 min prior to incubation with 5 μM LacCer for 8 h. Data represent one of the three separate experiments with similar results.

| Control | LacCer | NAC+LacCer | DPI+LacCer |
|---------|--------|------------|-----------|
| PMN Adhesion (Number of PMN/mm²) |
| Control | LacCer | NAC+LacCer | DPI+LacCer |
| 0  | 2.5  | 5  | 10  |
| 0  | 200  | 400  | 600  |

**Fig. 9. Effects of LacCer on the adhesion of neutrophils to HUVECs.** Endothelial cells were confluent grown in glass chamber slides. Cells were stimulated with 5 μM LacCer for 8 h followed by labeling with fluorescent (orange color) Cell Tracker dye for 30 min. Freshly prepared human neutrophils were stained with a green fluorescent (green color) Cell Tracker dye, coincubated with the endothelial cells for 20 min, washed with PBS, and visualized under fluorescent confocal microscopy as described under “Materials and Methods.” In a parallel experiment, endothelial cells were preincubated with either 5 μM DPI or 15 mM NAC for 30 min prior to incubation with 5 μM LacCer for 8 h. Data represent one of the three separate experiments with similar results.

**Fig. 10. Quantitative measurement of PMN adhesion to HUVECs.** A, endothelial cells were incubated with varying concentrations of LacCer (0–10 μM) for 4 h at 37 °C and washed with PBS. Freshly prepared PMNs labeled with Cell Tracker dye (green) were incubated with the endothelial cells for 30 min and washed, and the residual adherent PMNs were quantified using a fluorescence plate reader at 480 nm (excitation)/530 nm (emission) as described under “Materials and Methods.” ○, control; ●, LacCer; ▲, Ab; ▼, LacCer plus Ab. B, in a parallel experiment, LacCer-stimulated endothelial cells were incubated with various dilutions of ICAM-1 antibody followed by PMN adhesion. ○; Ab; ●, LacCer plus Ab. The data represent mean ± S.D. of three separate experiments.

**Fig. 11. Hypothetical model depicting a potential role of lactosylceramide as a lipid second messenger in TNF-α-induced ICAM-1 expression in HUVECs.**

...ferases (37–40) to assess whether the inhibition of LacCer synthesis could abrogate ICAM-1 expression. Indeed, as shown in Figs. 1 and 2, in endothelial cells t-PDMP not only inhibited the activity of GalT-2 and LacCer synthesis but also abrogated ICAM-1 expression. Since t-PDMP, an analog of glucosylceramide (GlcCer), also inhibits the synthesis of GlcCer from ceramide, it was not clear whether GlcCer or LacCer was required for the TNF-α-induced expression of ICAM-1. To address this issue, the following experiment was performed. First, we preincubated cells with t-PDMP followed by stimulation with TNF-α, GlcCer, and ceramide. LacCer was added next, and ICAM-1 expression was measured. Only LacCer bypassed the inhibitory effect of t-PDMP on ICAM-1 expression, while GlcCer or Cer did not. These observations suggest that LacCer may serve as a lipid second messenger in TNF-α-induced ICAM-1 expression.

In agreement with previous studies, we found that TNF-α up-regulates the expression of VCAM-1 and E-selectin in HUVECs. However, LacCer specifically stimulates the expression of ICAM-1 in these cells both at transcriptional and translational levels. At present, we cannot explain the biochemical basis of this observation. We can speculate, however, that TNF-α-mediated expression of VCAM-1 or E-selectin may require additional signaling molecules other than LacCer.

Previous studies have shown that TNF-α induces the expression of adhesion molecules via the generation of ROS (14, 15). We found that LacCer-induced ICAM-1 expression is also dependent on the generation of ROS. This tenet is supported by the following observations. First, direct measurement by lucigenin chemiluminescence showed that LacCer induces the generation of superoxide in a time- and concentration-dependent manner. We employed lucigenin chemiluminescence to assay LacCer-induced O2− generation, since this method detects both intracellular and extracellular O2− accurately even at a very low concentration (23, 31). Second, antioxidants NAC and pyrrolidine dithiocarbamate (41) and overexpression of superoxide dismutase gene abrogated ICAM-1 expression. Third, DPI, an inhibitor of the NADPH oxidase-dependent ROS-generating system (23), completely abrogated LacCer-induced ICAM-1 expression both at the transcriptional and translational level. Finally, TNF-α-induced O2− generation in HUVECs was abrogated by preincubation of cells with t-PDMP (data not shown). These findings suggest that TNF-α-induced LacCer production is necessary for the generation of O2−, and that in turn induces ICAM-1 expression.

Increased expression of ICAM-1 in atherosclerotic plaque has been previously thought to be due to increased O2− levels (15, 16, 42–44). Moreover, endothelial cells produce higher amounts of O2− in hypercholesterolemia (45). Since the level of LacCer is also elevated in atherosclerotic plaque intima (20, 21) and in patients with familial hypercholesterolemia (18, 19), it is tempting to speculate that this GSL may serve as a lipid second messenger that may be required to stimulate O2− production is necessary for the generation of O2−.
cific for NF-κB (data not shown). Previous studies have shown that the cytosolic NF-κB migrates to the nucleus and participates in nuclear events that ultimately lead to the expression of ICAM-1 (14). Clearly, further studies are required to explore in detail the LacCer-induced phenomenon described above.

The localized attachment of circulating leukocytes and monocytes to the endothelium of inflamed vessels is an important event in the initiation and progression of atherosclerotic lesion formation (47). As a response to injury, the endothelium secretes cytokines, for example, TNF-α, IL-8, platelet activation factor, and monocyte chemoattractant factor (2). Employing confocal microscopy, we found that LacCer facilitated the adhesion of PMNs to endothelial cells. These qualitative observations were substantiated by quantitative analysis of the number of PMNs adhering to the endothelial cells. Thus, LacCer may be implicated in the initiation of atherosclerotic plaque formation.

In summary, the adhesion of leukocytes to endothelial cells is a complex process, important in the pathogenesis of atherosclerosis and inflammatory phenomenon. Our findings indicate that TNF-α and oxidized low density lipoprotein implicated in atherosclerosis can activate GalT-2. TNF-α-mediated activation of GalT-2 can generate LacCer. LacCer, in turn, generates O2− and stimulates ICAM-1 expression in HUVECs. By virtue of stimulating the expression of the ligand Mac-1 (CD11/CD18) for ICAM-1 (36), LacCer facilitates the adhesion of leukocytes to activated endothelial cells that overexpress ICAM-1. This constitutes a novel biochemical mechanism in regard to the potential role of LacCer and ROS in TNF-α-induced initiation and progression of atherosclerosis.

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