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The Novel Neutrophil Differentiation Marker
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A new type of glycolipid, phosphatidylglucoside (PtdGlc), was identified as a component of raft-like membrane domains of the human leukemia cell line HL-60. In this study, we show that PtdGlc forms functional domains that are different from those produced by lactosylceramide (LacCer)-enriched lipid rafts. These rafts initiate neutrophil apoptosis. Neutrophils are the only type of human peripheral blood leukocyte or monocyte-derived dendritic cell to express large amounts of PtdGlc on their cell surfaces. PtdGlc was not colocalized with LacCer. Anti-PtdGlc IgM DIM21 did not induce neutrophil chemotaxis or superoxide generation, whereas anti-LacCer IgM TS5A7 induced these activities. DIM21, but not TS5A7, significantly induced neutrophil apoptosis. DIM21-induced apoptosis was inhibited by specific inhibitors of cysteine-containing aspartate-specific proteases (caspases)-8, -9, and -3 but not by the Src family kinase inhibitor PP1, PIP3 kinase inhibitor LY294002, NADPH oxidase inhibitor diphenyleneiodonium, superoxide dismutase, or catalase. PtdGlc was colocalized with Fas on the neutrophil plasma membrane. DIM21 and the agonist anti-Fas Ab DX2 induced the formation of large Fas-colocalized clusters of PtdGlc on the plasma membrane. Furthermore, the antagonistic anti-Fas Ab ZB4 significantly inhibited DIM21-induced neutrophil apoptosis. These results suggest that PtdGlc is specifically expressed on neutrophils and mediates apoptosis of these cells, and that the Fas-associated death signal may be involved in PtdGlc-mediated apoptosis. The Journal of Immunology, 2011, 186: 5323–5332.

Polymorphonuclear neutrophils play critical roles in the innate immune system by recognizing pathogen-associated molecular patterns via pattern recognition receptors expressed on the cell surface and then phagocytosing and eliminating microorganisms (1). Neutrophils have a very short lifespan and undergo apoptosis within 24–48 h after leaving the bone marrow.

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K.K., H.M., and H.N. performed experiments; Y.N. provided Abs and performed mass spectrometry analysis; T.N. performed mass spectrometry analysis; Y.H. provided Abs and analyzed data; Y.T., K.S., and H.D. performed flow cytometric analysis; H.O. and K.T. analyzed data; and K.I. designed and performed experiments.

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The online version of this article contains supplemental material.

Abbreviations used in this article: Alexa Fluor 488-DIM21, Alexa Fluor 488-conjugated DIM21; Alexa Fluor 647-TS5A7, Alexa Fluor 647-conjugated TS5A7; C/M, chloroform/methanol; DC, dendritic cell; DIc, differential interference contrast; DMEM/F12-BSA, DMEM/F12 medium supplemented with 1 mg/ml BSA; DMEM/F12-FBS, DMEM/F12 medium supplemented with 10% FBS; DPl, diphenylenoiodonium; LacCer, lactosylceramide; LC/MS/MS, liquid chromatography-tandem mass spectrometry; MS, mass spectrometry; PI, propidium iodide; PLP, periodate-lysine paraformaldehyde; PtdGlc, phosphatidylglucoside; ROS, reactive oxygen species; SOD, superoxide dismutase.

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phorylation of Lyn and Hck. Reduction of endogenous cholesterol with methyl-β-cyclodextrin suppresses anti-PtdGlc Ab-stimulated tyrosine phosphorylation, resulting in the up- and downregulation of CD38 and c-Myc expression, respectively (16). Therefore, PtdGlc may form functional raft-like domains on the plasma membranes of HL-60 cells, although it does not have C2 fatty acid chains (17). PtdGlc forms distinct lipid domains from sphingo-lipids on the outer leaflet of the plasma membrane of HL-60 cells (18). These observations suggest that PtdGlc and LacCer form lipids on the outer leaflet of the plasma membrane of HL-60 cells and may contribute to the differential signaling mechanisms.

This study demonstrated that PtdGlc is highly expressed on human neutrophils and is located in lipid domains distinct from LacCer-enriched lipid rafts in these cells. In addition, PtdGlc, but not LacCer, was involved in neutrophil apoptosis, which was mediated through Fas-dependent signal transduction.

**Materials and Methods**

**Abs and other reagents**

Mouse anti-PtdGlc IgM DIM21 and mouse anti-LacCer IgM TS47 were prepared as described (5, 17, 19). Mouse anti-human Fas (CD95) IgM CH-11 and IgG ZB4 were obtained from Medical and Biological Laboratories (Nagoya, Japan). Mouse PE-conjugated anti-CD11b IgG ICCR4, PerCP- Cy5.5-anti-CD14 IgG 61D3, and PE-CD54 IgG HAS8 were purchased from eBioscience (San Diego, CA). Mouse anti-Fas IgG DX2 and mouse anti-CD15s IgG CSLEX1 were from BD Biosciences (San Jose, CA). Complete protease inhibitor mixture (Complete) was obtained from Roche (Indianapolis, IN). PPI was obtained from Bioeml Research Laboratories (Plymouth Meeting, PA). Ly294002, cysteine-containing aspartate-specific protease 3 (caspase-3) inhibitor VII, caspase-8 inhibitor II, caspase-9 inhibitor III, and superoxide dismutase (SOD) from bovine erythrocytes were obtained from Calbiochem (La Jolla, CA). Catalase (from bovine liver) was from Wako Pure Chemical Industries, (Osaka, Japan). IMPL and PMA were from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

**Cell preparation and culture**

Human neutrophils were isolated from heparinized peripheral blood of healthy volunteers, with their informed consent, using Polymorphprep (Nycomed Pharma, Oslo, Norway), centrifuged as described previously (6), and suspended at 107 cells/ml in DMEM/F12 medium. The neutrophil population was >95% pure, as determined by Wright–Giemsa staining, and viability was always >99%, as determined by trypan blue dye exclusion (Wako).

Monocyte-derived dendritic cells (DCs) were prepared from peripheral blood as described previously, with slight modifications (20). Briefly, mononuclear cells were isolated from peripheral human blood using Lym- phoprep (Nycomed Pharma, Oslo, Norway), and monocytes were isolated from mononuclear cells using a monoclonal antibody kit (Miltenyi Biotec, Bergisch Gladbach, Germany). DCs were differentiated from monocytes by incubation in RPMI 1640 medium supplemented with 10% autologous human serum, 100 U/ml GM-CSF (R&D Systems, Minneapolis, MN), and 50 ng/ml IL-4 (R&D Systems) in a CO2 incubator for 3 d.

**Liquid chromatography-tandem mass spectrometry analysis**

Neutrophils were extracted twice with chloroform/methanol (1:1, v/v) and twice with chloroform/methanol/water (5:8:3, v/v/v) and then the extracts were dried under a stream of nitrogen gas. The dried extract was treated twice with propidium iodide (PI)-specific phospholipase C (Sigma-Alrich). The resulting reaction mixture was lyophilized, and PtdGlc was further purified on an HPLC (JASCO, Hachioji, Japan), using a Mono A column and suspended at 107 cells/ml in DMEM/F12 medium. The neutrophil population was >95% pure, as determined by Wright–Giemsa staining, and viability was always >99%, as determined by trypan blue dye exclusion (Wako).

**Flow cytometric analysis**

The expression of cell-surface Ags was assessed by flow cytometry using a FACS Calibur (BD Biosciences), as described (5). Briefly, neutrophils were stained with Alexa Fluor 488-anti-PtdGlc IgM DIM21, Alexa Fluor 647-anti-LacCer IgM TS47, or Alexa Fluor-labeled normal IgM for 30 min on ice. In some experiments, neutrophils were incubated with or without TS47 or DIM21 at 37°C for 15 min, followed by staining with Alexa Fluor 488-conjugated DIM21 (Alexa Fluor 488-DIM21) or Alexa Fluor 647-conjugated TS47 (Alexa Fluor 647-TS47) on ice for 30 min, respectively. In some experiments, neutrophils were incubated at 37°C for 30 min before staining with Abs on ice for 30 min.

**Immunostaining**

Aliquots of 2 × 106 neutrophils were fixed with periodate-lysine paraformaldehyde (PLP) fixative solution (2% paraformaldehyde, 0.1 M glycine, and 0.2% sodium periodate in 50 mM phosphate buffer [pH 7.4]) for 30 min on ice. The cells were washed and blocked with incubation with 0.1% BSA and 5% normal goat serum in PBS for 60 min on ice. Aliquots of cells were permeabilized with 5 μg/ml digitonin for 10 min on ice and blocked with 0.1% BSA and 5% normal goat serum. The cells were stained with Alexa Fluor 546-DIM21 and Alexa Fluor 488-TS47 for 30 min on ice. The stained cells were examined with a TCS-S2 Leica confocal microscope equipped with a Plan-Apochromat 100× oil-immersion differential interference contrast (DIC) objective. Images were evaluated using multicolor/two-dimensional cytofluorogram software from Leica Microsystems. The software quantifies the extent of colocalization by creation of a binary mask of the image data in the cytofluorogram. The binary mask is created by masking all of the pixels that are double positive for Alexa Fluor 546-DIM21 and Alexa Fluor 488-TS47 fluorescence. Colocalization was then assessed using the mask intensity rate for the colocalized Alexa Fluor 546 versus the overall intensities of the Alexa Fluor 546 in the image. A mask intensity rate >50% was used to confirm colocalization.

To assess the effects of anti-PtdGlc IgM DIM21 and anti-Fas IgG DX2 on the formation of PtdGlc and Fas clusters on the plasma membrane, neutrophils were incubated with or without 5 μg/ml anti-Fas IgG DX2, anti-PtdGlc IgM DIM21, or anti-sialyl Le α IgM CSLEX1 at 37°C for 5 min, followed by additional incubation for 25 min on ice. As negative controls, neutrophils were incubated at 37°C for 5 min without Ab (−) and then incubated for an additional 25 min on ice with DIM21. The cells were washed three times with ice-cold PBS, fixed with PLP fixative solution for 30 min on ice, blocked with 0.1% BSA and 5% normal goat serum in PBS, incubated with DIM21 or DX2 for 30 min on ice, and then stained with fluorescent-conjugated secondary Abs.

**Migration assay**

Cell migration was assessed using a modified Boyden chamber with cellulose nitrate filters with a pore size of 3 μm (Sartorius, Göttingen, Germany), as described (8). Briefly, 0.1 × 106 cells/ml in DMEM/F12-BSA were placed into the upper compartment and incubated for 15 min, followed by additional incubation for 25 min on ice. As negative controls, neutrophils were incubated at 37°C for 5 min without Ab (−) and then incubated for an additional 25 min on ice with DIM21. The cells were washed three times with ice-cold PBS, fixed with PLP fixative solution for 30 min on ice, blocked with 0.1% BSA and 5% normal goat serum in PBS, incubated with DIM21 or DX2 for 30 min on ice, and then stained with fluorescein-conjugated secondary Abs.

**Superoxide generation assay**

Superoxide generation from neutrophils was assessed by measuring SOD-inhibited cytchrome c reduction, as described (5). Aliquots of 100 μl of neutrophils (2.5 × 105 cells/100 μl) in DMEM/F12-BSA were incubated with or without 50 μM SOD for 30 min at 37°C. The neutrophils had been precoated with DIM21, TS47, or normal IgM, in a CO2 incubator. As control experiments, neutrophils were incubated with 500 nM fMLP or 0.1% DMSO in BSA-coated wells at 37°C for 30 min. The
absorbance of the supernatants at 550 nm was measured with a Beckman DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA), and cytochrome c reduction was calculated using the formula: F_red = 2.1 × 10^4 M^−1 cm^−1 (23).

**Annexin V-binding assay**

Neutrophils (1 × 10^6 cells/ml) in DMEM/F12 medium supplemented with 10% FBS (DMEM/F12-FBS) were incubated with 4 or 8 μg/ml DIM21, TS57, or normal IgM for 1 min, 1 h, and 4 h at 37°C in a CO2 incubator. The apoptotic activity of DIM21 on neutrophils was sometimes changed by batch-to-batch variation. Moreover, the biological activity of DIM21 is not stable. Thus, the optimal working concentration of DIM21 was adjusted in each experiment. Usually, the submaximal concentration showing the maximum apoptotic activity of DIM21 was 8 μg/ml. In some experiments, neutrophils were preincubated for 30 min on ice with caspase-3 inhibitor VII (50 μM), caspase-8 inhibitor II (50 μM), caspase-9 inhibitor III (50 μM), PP1 (2 μM), or LY294002 (2 μM).

To determine the involvement of reactive oxygen species (ROS) in DIM21-induced neutrophil apoptosis, neutrophils were preincubated with 10 μM diphenyleneiodonium (DPI) for 30 min at 37°C in DMEM/F12-FBS (24). The loaded cells were then incubated with 4 μg/ml normal IgM or anti-PtdGlc DIM21 at 37°C for 4 h. To determine the effects of catalase on anti-PtdGlc Ab-induced apoptosis, neutrophils were incubated with DIM21 in the absence or presence of 140 U/ml SOD or 200 U/ml catalase at 37°C for 4 h. To determine the effects of anti-Fas Ab ZB4 on DIM21-induced neutrophil apoptosis, neutrophils (2.5 × 10^6 cells/ml) were preincubated with 10 μg/ml ZB4 for 1 h on ice prior to DIM21 treatment.

The binding of Alexa Fluor 488-conjugated Annexin V to cells was used as a sensitive method for measuring apoptosis (25). Specific binding of Annexin V was determined by Alexa Fluor 488-conjugated Annexin V and PI for Apoptosis Detection Kit (Invitrogen), according to the manufacturer’s instructions. The Annexin V-bound cells were analyzed using a FACSCalibur flow cytometer. The binding assay indicated that Annexin V did not bind to DIM21 (Supplemental Fig. 1).

**TUNEL assay**

The TUNEL assay with fluorescein-dUTP, using an In situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN), was used to detect and quantify DNA strand breaks in apoptotic neutrophils, according to the manufacturer’s instructions. The stained cells were examined with a TCS-SP2 Leica confocal microscope equipped with a Plan-Apochromat 63× oil-immersion DIC objective.

**Colorimetric measurement of caspase-3 activity**

Neutrophils (2 × 10^6 cells/ml) were incubated with 1 μg/ml DIM21, normal IgM, or TS57 in DMEM/F12-FBS at 37°C for 4 h in a CO2 incubator. The caspase-3 activity of the cells was analyzed using an ApoAlert caspase assay kit (Takara Bio, Shiga, Japan), according to the manufacturer’s instructions.

**Statistical analysis**

The data are expressed as the means ± SD, and significant differences were analyzed by one-way ANOVA and post hoc test using the GraphPad PRISM statistical software package (GraphPad Software, San Diego, CA). In all analyses, p < 0.05 indicated statistical significance.

**Results**

PtdGlc is preferentially expressed on neutrophils

PtdGlc is a new type of glycolipid (16) that is preferentially expressed along the neutrophil-differentiation pathway (26). Consistent with these findings, we observed that PtdGlc was highly expressed on the cell surfaces of peripheral blood neutrophils (CD14\(^{low}\) and CD11b\(^{low}\)) and weakly expressed on the cell surfaces of monocytes (CD11b\(^{high}\) and CD14\(^{high}\) cells) and lymphocytes (CD54\(^{+}\) and CD14\(^{−}\) cells; Fig. 1). Neutral glycosphingolipid LacCer has been identified as the neutrophil-differentiation marker CDw17 (27). Most cells highly expressing LacCer also showed high levels of PtdGlc expression (Fig. 1B). Peripheral blood-derived unstimulated DCs expressed LacCer, but did not express PtdGlc, on their cell surfaces (Fig. 1A). The presence of PtdGlc on neutrophils was also confirmed by liquid chromatography/MS analyses (Fig. 2). PtdGlc was detected using a nano liquid chromatography-tandem mass spectrometry (LC/MS/MS) system after partial purification. Extracted ion current chromatogram by m/z = 893.6, which is the mass of a single molecular species of fetal murine brain PtdGlc, showed a peak at T12.6 (Fig. 2A). However, in the case of neutrophils, PtdGlc contained C18:0 fatty acyl chain as a major component. In addition, a peak with m/z of 935.6, identical to 6-O-acetylated, C18:0, 20:0-PtdGlc was also found at T10.3. A peak with m/z of 907.6 corresponding to 6-O-acetylated PtdGlc with C18:0, 18:0 was also found at T10.7, but the ratio relative to PtdGlc was less than that of C18:0, 20:0-PtdGlc. These molecular species were confirmed as PtdGlc-related compounds by MS and LC/MS/MS spectra (Fig. 2B). The fragmentation profile is comparable to previous results (28). LacCer was found to be the major glycolipid component of neutrophils, whereas PtdGlc content was ~0.75% of the lactosylceramide content estimated from the liquid chromatography/MS peak area.

**PtdGlc forms domains that differ from LacCer-enriched microdomains**

Human neutrophils abundantly express LacCer on their cell surfaces, and LacCer clusters to form lipid rafts coupled with Lyn on the plasma membranes of neutrophils and HL-60 cells (5). PtdGlc also forms raft-like membrane domains and activates Lyn in HL-
Neutrophils are short-lived cells that survive in the circulation for ~24–36 h before undergoing apoptosis (31). Therefore, neutrophil turnover is rapid, and the efficient removal of such large numbers of effete cells by apoptosis is a major homeostatic endeavor (7). PtdGlc was already shown to be expressed on myelocytes, and anti-PtdGlc Ab was able to induce the differentiation of HL-60 cells to neutrophils. Therefore, we first examined the effects of anti-PtdGlc IgM DIM21 on Annexin V expression in neutrophils to determine whether PtdGlc induces apoptosis of neutrophils, because Annexin V is a sensitive probe used to quantify the cells that undergo apoptosis (32). DIM21 treatment induced Annexin V+ cells in an incubation time-dependent manner (Fig. 4). In contrast, anti-LacCer Ab did not enhance neutrophil apoptosis. Human monoclonal IgM GL-2, which specifically reacts with PtdGlc (13), and recombinant GL-2 Fab fragment rGL-7 also induced Annexin V+ cells (Fig. 4C). PI positivity indicates necrotic cells. As shown in Fig. 4A, the percentages of PI+ DIM21-treated cells (~6% of total after 4 h of incubation) were almost the same as those of other Ab-treated cells. Thus, DIM21 was unlikely to have induced necrosis of neutrophils. The TUNEL reaction is also used to measure apoptosis, because DNA cleavage generally occurs at a much greater frequency in apoptotic cells than in nonapoptotic cells. Therefore, we tested the apoptotic effect of DIM21 on neutrophils by the TUNEL method. Neutrophils with typical apoptotic morphology, including rounding of nuclei, were observed after 4 h of incubation with DIM21, and these cells were TUNEL+ (Fig. 5A). As shown in Fig. 5C, DIM21-induced TUNEL+ cells were significantly increased by DIM21 treatment (26.8 ± 4.6%, mean ± SD of three experiments), but not by T5A7 treatment (19.0 ± 5.4%), compared with those in normal IgM-treated cells (14.9 ± 5.6%). Moreover, caspase-3 activation, which is another hallmark event leading to apoptosis (33), was significantly induced by treatment with DIM21 for 4 h but not by T5A7 (Fig. 5D). These observations suggested that PtdGlc mediates neutrophil apoptosis, whereas LacCer does not participate in this process.

**PtdGlc-induced apoptosis is dependent on caspases but not Src family kinase, PIP3 kinase, or superoxide generation**

Despite the numerous available apoptotic stimuli, the final changes in the cells are similar, and many of the signaling events seem to

**FIGURE 2.** MS of PtdGlc in normal human neutrophils. A, MS chromatograms of PtdGlc. Total lipid extract from normal human neutrophils (10⁶) was pretreated with PI-specific phospholipase C to remove PI. The major glycolipid, LacCer, was also separated from PtdGlc by HPLC equipped with a MonoBis normal phase column. The partially purified sample was then analyzed using a nano LC/MS/MS system. a, Total ion chromatogram; b–e, MS chromatogram scanned with m/z of 893.6 (b, PtdGlc with 18:0, 20:0), 865.6 (c, PtdGlc with 18:0, 18:0), 935.6 (d, 6-O-Ac-PtdGlc with 18:0, 18:0), and 907.6 (e, 6-O-Ac-PtdGlc with 18:0, 18:0). The results shown are representative data of two experiments. b, MS spectra (a, b) and LC/MS/MS spectra (c–e) of neutrophil PtdGlc-related molecules. PtdGlc (a) and 6-O-Ac-PtdGlc (b) production spectrum of PtdGlc with 18:0, 20:0 (c) and 18:0, 20:0 (d) and 6-O-Ac-PtdGlc with 18:0, 20:0 (e).

contrast, PtdGlc was upregulated in a temperature-dependent manner. The upregulated expression of PtdGlc was not downregulated by cooling on ice (Supplemental Fig. 2). These observations suggested that, as in the case of αsβ2 integrin, PtdGlc exists in the granules, which are translocated to the plasma membrane in a temperature-dependent manner. Although anti-LacCer Ab T5A7 activates signal-transduction molecules, including Lyn and p38 MAPK, T5A7 did not enhance the expression of PtdGlc on the cell surface.

LacCer mediates neutrophil chemotaxis and the generation of superoxide from neutrophil-differentiated HL-60 cells with a bell-shaped dose-response curve from 0.01–10 μg/ml (8). Therefore, we also examined the effects of DIM21 on neutrophil chemotaxis and the generation of superoxide to determine whether PtdGlc is also involved in these LacCer-mediated activities. However, we found that DIM21 minimally enhanced migration (Fig. 3F) or superoxide generation (Fig. 3G), suggesting that PtdGlc is not involved in LacCer-mediated neutrophil functions.

### PtdGlc is involved in neutrophil apoptosis

Neutrophils with typical apoptotic morphology, including rounding of nuclei, were observed after 4 h of incubation with DIM21, and these cells were TUNEL+ (Fig. 5A). As shown in Fig. 5C, DIM21-induced TUNEL+ cells were significantly increased by DIM21 treatment (26.8 ± 4.6%, mean ± SD of three experiments), but not by T5A7 treatment (19.0 ± 5.4%), compared with those in normal IgM-treated cells (14.9 ± 5.6%). Moreover, caspase-3 activation, which is another hallmark event leading to apoptosis (33), was significantly induced by treatment with DIM21 for 4 h but not by T5A7 (Fig. 5D). These observations suggested that PtdGlc mediates neutrophil apoptosis, whereas LacCer does not participate in this process.

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Neutrophils are short-lived cells that survive in the circulation for ~24–36 h before undergoing apoptosis (31). Therefore, neutrophil turnover is rapid, and the efficient removal of such large numbers of effete cells by apoptosis is a major homeostatic endeavor (7). PtdGlc was already shown to be expressed on myelocytes, and anti-PtdGlc Ab was able to induce the differentiation of HL-60 cells to neutrophil cells, suggesting that PtdGlc may be involved in differentiation-related events, including apoptosis, in neutrophils. Therefore, we first examined the effects of anti-PtdGlc IgM DIM21 on Annexin V expression in neutrophils to determine whether PtdGlc induces apoptosis of neutrophils, because Annexin V is a sensitive probe used to quantify the cells that undergo apoptosis (32). DIM21 treatment induced Annexin V+ cells in an incubation time-dependent manner (Fig. 4). In contrast, anti-LacCer Ab did not enhance neutrophil apoptosis. Human monoclonal IgM GL-2, which specifically reacts with PtdGlc (13), and recombinant GL-2 Fab fragment rGL-7 also induced Annexin V+ cells (Fig. 4C). PI positivity indicates necrotic cells. As shown in Fig. 4A, the percentages of PI+ DIM21-treated cells (~6% of total after 4 h of incubation) were almost the same as those of other Ab-treated cells. Thus, DIM21 was unlikely to have induced necrosis of neutrophils. The TUNEL reaction is also used to measure apoptosis, because DNA cleavage generally occurs at a much greater frequency in apoptotic cells than in nonapoptotic cells. Therefore, we tested the apoptotic effect of DIM21 on neutrophils by the TUNEL method. Neutrophils with typical apoptotic morphology, including rounding of nuclei, were observed after 4 h of incubation with DIM21, and these cells were TUNEL+ (Fig. 5A). As shown in Fig. 5C, DIM21-induced TUNEL+ cells were significantly increased by DIM21 treatment (26.8 ± 4.6%, mean ± SD of three experiments), but not by T5A7 treatment (19.0 ± 5.4%), compared with those in normal IgM-treated cells (14.9 ± 5.6%). Moreover, caspase-3 activation, which is another hallmark event leading to apoptosis (33), was significantly induced by treatment with DIM21 for 4 h but not by T5A7 (Fig. 5D). These observations suggested that PtdGlc mediates neutrophil apoptosis, whereas LacCer does not participate in this process.
converge into common mechanisms involving activation of caspases (34). Death receptors, such as Fas, transmit death signals after stimulation by their specific ligands. Stimulation of death receptors leads to activation of the initiator caspase-8, which sequentially activates effector caspases, such as caspase-3, leading to apoptotic cell death (35). Caspase-3 is the final downstream effector caspase mediating apoptosis in neutrophils (36). Functional inhibitor studies suggested that caspase-9 is crucial for caspase-3 and -8 activation, at least in neutrophils (37). Therefore, we examined the involvement of these caspases in PtdGlc-mediated neutrophil apoptosis. As shown in Fig. 6A, specific inhibitors of caspases-3, -8, and -9 significantly inhibited DIM21-

FIGURE 3. PtdGlc is present in different domains from LacCer-enriched lipid rafts. A, Effects of T5A7 on the expression of PtdGlc. Neutrophils were incubated with 1 μg/ml T5A7 or DIM21 on ice for 30 min, fixed, and stained with Alexa Fluor 488-DIM21 (PtdGlc) or Alexa Fluor 647-T5A7 (LacCer), respectively. Dashed lines indicate normal IgM-treated cells. Numbers represent the geometric means of fluorescence intensity. B, Colocalization of PtdGlc and LacCer on neutrophils. Neutrophils were fixed with PLP fixative solution for 30 min on ice and then treated (Digitonin +) or not (Digitonin –) with 5 μg/ml digitonin for 10 min. After washing, the cells were incubated with Alexa Fluor 488-conjugated anti-LacCer T5A7 IgM (green) and Alexa 546-conjugated anti-PtdGlc DIM21 IgM (red). The stained cells were examined with a TCS-SP2 Leica confocal microscope equipped with a Plan-Apochromat 100× oil-immersion DIC objective. Scale bar, 10 μm. The data shown are representative data of three experiments. C, Fluorescence intensity profiles of plasma membrane area in image overlays shown in Bb (left panel). Right panel, Cytofluorogram from Alexa Fluor 488-conjugated anti-LacCer T5A7 (LacCer) and Alexa Fluor 546-conjugated anti-PtdGlc DIM21 (PtdGlc) in image overlays shown in the left panel. The mask intensity rate was 6.5%. D, Cytofluorogram from Alexa Fluor 488-T5A7 (LacCer) and Alexa Fluor 546-anti-PtdGlc DIM21 (PtdGlc) in image overlays shown in Bb. The mask intensity rate was 0.2%. E, Temperature-dependent upregulation of PtdGlc. Neutrophils were incubated with or without (–) 1 μg/ml DIM21 (DIM) or T5A7 at 37°C for 15 min. Some cells were incubated without Abs on ice for 15 min (On ice). The cells were then stained with Alexa Fluor 647-T5A7 (a), Alexa Fluor 488-DIM21 (b), or PE–anti-CD11b (c) on ice for 30 min. The cells were washed, and the binding of each Ab to neutrophils was analyzed by flow cytometry. BG, Cells were treated with normal IgM on ice and stained with Alexa Fluor 488–, Alexa Fluor 488–, or PE-conjugated anti-IgM. Numbers are the geometric means of fluorescence intensity of neutrophils without Ab incubating on ice (On ice) or at 37°C (0.37°C (–)). F, Effects of anti-PtdGlc Ab on neutrophil chemotaxis. Anti-PtdGlc Ab DIM21, anti-LacCer Ab T5A7, and normal IgM were assessed for chemotactic activities against neutrophils by the Boyden chamber method. IgM, 0.1 μg/ml normal IgM; T5A7, 0.1 μg/ml anti-LacCer Ab T5A7; DMSO, solvent control for fMLP stimulation; fMLP, 10 nM fMLP. Each bar shows the mean ± SD of four independent experiments. ***p < 0.001. G, Effects of anti-PtdGlc Ab on neutrophil superoxide generation. The superoxide generation assay was performed as described in Materials and Methods. **p < 0.01, ***p < 0.001. IgM, 10 μg/ml normal IgM; T5A7, 10 μg/ml anti-LacCer Ab T5A7; DMSO, solvent control for fMLP stimulation; fMLP, 500 nM fMLP. Each bar shows the mean ± SD of three independent experiments.
induced neutrophil apoptosis. Therefore, cross-linking of PtdGlc likely induces neutrophil apoptosis via the activation of caspases-8, -9, and -3.

In the case of LacCer-enriched lipid rafts, the activation of Src family kinases and PIP3 kinases is thought to play crucial roles in LacCer-mediated signal transduction (1, 38). PtdGlc was demonstrated to form lipid raft-like domains and to be involved in rapid tyrosine phosphorylation of Src family protein kinases Lyn and Hck (16). Therefore, we examined the effects of the Src family kinase inhibitor PP1 and PIP3 kinase inhibitor LY294002 on DIM21-induced neutrophil apoptosis. As shown in Fig. 6B, the Src family kinase inhibitor PP1 (2 μM) or the specific PIP3 kinase inhibitor LY294002 (2 μM) did not affect DIM21-induced neutrophil apoptosis, whereas these concentrations completely inhibited LacCer-mediated neutrophil functions (6). Even a high concentration (10 μM) of PP1 or LY294002 and mixtures of these compounds did not affect DIM21-induced neutrophil apoptosis (data not shown). Lyn and PIP3 kinase were shown to be involved in neutrophil survival (39, 40). Therefore, it is unlikely that Src family kinases, including Lyn, and PIP3 kinases are involved in PtdGlc-mediated neutrophil apoptosis.

Several studies indicated that ROS are involved in neutrophil apoptosis (36, 41). The accumulation of ROS may initiate ceramide generation and the clustering of preformed Fas-inducing signaling complexes in lipid rafts (42). SOD enhances spontaneous neutrophil apoptosis, whereas catalase inhibits their spontaneous apoptosis and caspase-3 activation, suggesting that hydrogen peroxide is a potential mediator of ROS-induced neutrophil apoptosis in a caspase-dependent manner (43). Indeed, neutrophils from patients with chronic granulomatous disease, who

**FIGURE 4.** Effects of anti-PtdGlc Ab on neutrophil apoptosis. A, Neutrophils were incubated without (Control) or with 8 μg/ml anti-PtdGlc Ab DIM21, anti-LacCer Ab T5A7, or normal IgM at 37˚C for 1 min, 1 h, and 4 h. The cells were then fixed and incubated with Alexa Fluor 488-conjugated Annexin V for 15 min at room temperature, stained with PI, and analyzed by flow cytometry. Representative results of three independent experiments are shown. B, Annexin V+ and PI+ cells were gated as shown in A, and the numbers of Annexin V+ cells were calculated. Each point represents the mean ± SD of three independent experiments. *p < 0.05, ***p < 0.001. C, Neutrophils were incubated with 8 μg/ml normal IgM or anti-PtdGlc Ab DIM21 at 37˚C for 1 min or 4 h. After incubation, cells were cytospun onto slide glasses and fixed with 4% paraformaldehyde, followed by staining with fluorescein-dUTP using an In situ Cell Death Detection Kit, as described in the Materials and Methods. Scale bars, 10 μm.

**FIGURE 5.** PtdGlc involvement in neutrophil apoptosis. A, Neutrophils were incubated with 8 μg/ml normal IgM or anti-PtdGlc Ab DIM21 at 37˚C for 4 h. After incubation, cells were cytospun onto slide glasses and fixed with 4% paraformaldehyde, followed by staining with fluorescein-dUTP using an In situ Cell Death Detection Kit, as described in the Materials and Methods. Scale bars, 10 μm. B, Neutrophils were incubated with 8 μg/ml anti-PtdGlc Ab DIM21, anti-LacCer Ab T5A7, or normal IgM at 37˚C for 1 min or 4 h. After incubation, cells were fixed with 4% paraformaldehyde. The cells with chromatin fragmentation were then stained with the chromatin fragmentation kit. The cells were examined with a TCS-SP2 Leica confocal microscope with a Plan-Apochromat 63× oil-immersion DIC objective. Scale bars, 10 μm. C, The TUNEL+ cells, as shown in B, were counted. Data are shown as the means ± SD of three independent experiments. *p < 0.01. D, Effects of anti-PtdGlc Ab on caspase-3 activity. Neutrophils were incubated with 8 μg/ml anti-PtdGlc Ab DIM21, anti-LacCer Ab T5A7, or normal IgM at 37˚C for 4 h, and caspase-3 activity of the cells was analyzed as described in Materials and Methods. Data are shown as the means ± SD of three independent experiments. ***p < 0.001.
have a deficiency in NADPH oxidase activity, also undergo spontaneous apoptosis but have a prolonged lifespan (44). However, another group could not detect a difference in the rate of spontaneous apoptosis between normal and NADPH oxidase-deficient neutrophils (7). Neither SOD nor catalase affected DIM21-induced neutrophil apoptosis under our experimental conditions (Fig. 6C, 6D). DPI, which is a strong NADPH oxidase inhibitor, did not inhibit DIM21-induced apoptosis, although it completely inhibited PMA-induced superoxide generation from neutrophils (Fig. 6E, 6F). DIM21 did not induce superoxide generation by itself (Fig. 3G). These observations suggested that it is unlikely that NADPH oxidase-mediated ROS is involved in PtdGlc-mediated neutrophil apoptosis.

Fas is involved in PtdGlc-mediated apoptosis

Fas is a death receptor associated with lipid rafts during Fas-mediated apoptosis (45–47). Therefore, we examined whether PtdGlc is colocalized with Fas in neutrophils. PtdGlc and Fas were colocalized on the plasma membranes in resting neutrophils. PtdGlc formed large clusters and was colocalized with Fas following stimulation with anti-PtdGlc IgM DIM21 (Fig. 7A). Moreover, treatment with anti-Fas IgG DX2, which can induce apoptosis, caused the formation of Fas clusters, which were also colocalized with PtdGlc (Fig. 7B). The mask intensity rates of resting, DIM21-treated, or DX2-treated neutrophils were 85.7, 89.5, and 90.4%, respectively (Fig. 7C). Neutrophils also expressed high levels of CD15s (sialyl Leα Ag) on their cell surfaces. However, treatment with anti-CD15s IgM did not induce Fas-colocalized cluster formation. The mask intensity rate of anti-CD15s IgM-treated neutrophils was only 1.7%. These observations suggested that PtdGlc forms large clusters with Fas along with activating cells through the cross-linking of PtdGlc or Fas. Fas is activated by inducing its trimerization (48). Activated Fas recruits adaptor molecules, such as FADD, which recruit procaspase-8 to the receptor complex where it undergoes autocatalytic activation. Incubation of neutrophils with agonistic anti-Fas Abs significantly accelerates the apoptotic process in these cells (25, 49). The Fas agonistic Ab CH-11 was presumed to mediate its apoptotic effect through cross-linking of Fas receptors, in a manner analogous to the Fas ligand. In contrast, the Fas antagonistic Ab ZB4 can effectively block apoptosis induced by CH-11 by binding to the same epitope as CH-11 (50). ZB4 inhibits Fas ligation and efficiently blocks apoptotic cell death induced by the agonistic anti-Fas IgM CH-11 or Fas ligand in human neutrophils (51). We found that ZB4 significantly inhibited CH-11- and DIM21-induced neutrophil apoptosis (Fig. 7E). Therefore, the Fas-mediated activation of FADD seems to be involved in PtdGlc-mediated neutrophil apoptosis.

Discussion

More than 400 species of glycolipid have been identified based on the sugar chain structures (52). Glycolipids have attracted considerable interest because leukocytes show lineage-related and differentiation-dependent expression of these molecules, making

![FIGURE 6. Effects of inhibitors of signal transduction molecules on PtdGlc-mediated apoptosis. A. Effects of caspase inhibitors on anti-PtdGlc Ab-induced apoptosis. Neutrophils were preincubated with 50 μM caspase-3 inhibitor VII, caspase-8 inhibitor II, or caspase-9 inhibitor III for 30 min on ice. The apoptosis ratio of each experiment was calculated as (percentage of DIM21- or normal IgM-induced apoptotic cells in the inhibitor-treated cells)/percentage of DIM21-induced apoptotic cells). Each bar shows the mean ± SD of three independent experiments. B. Anti-PtdGlc Ab-induced apoptosis was not mediated by Src family kinases or PIP3 kinases. Neutrophils were incubated with DMSO (as solvent control), 2 μM PP1, or LY294002 for 30 min on ice. These cells were incubated without or with 1 μg/ml normal IgM or anti-PtdGlc DIM21 for 4 h at 37°C, and specific binding of Alexa Fluor 488-conjugated Annexin V to neutrophils was analyzed by flow cytometry. Each bar shows the mean ± SD of three independent experiments. C. Effects of SOD on anti-PtdGlc Ab-induced apoptosis. Neutrophils were incubated without or with 4 μg/ml normal IgM or anti-PtdGlc Ab DIM21 in the absence (SOD −) or presence (SOD +) of 140 U/ml of SOD at 37°C for 4 h. Specific binding of Alexa Fluor 488-conjugated Annexin V to neutrophils was analyzed by flow cytometry. Representative results of three independent experiments are shown. The numbers in each panel are the percentages of Annexin V+ cells. Each bar shows the mean ± SD of three independent experiments. D. Effects of catalase on anti-PtdGlc Ab-induced apoptosis. Neutrophils were incubated without or with 4 μg/ml normal IgM or anti-PtdGlc Ab DIM21 in the absence (catalase −) or presence (catalase +) of 200 U/ml catalase at 37°C for 4 h. Specific binding of Alexa Fluor 488-conjugated Annexin V to neutrophils was analyzed by flow cytometry. Representative results of three independent experiments are shown. The numbers in each panel are the percentages of Annexin V+ cells. Each bar shows the mean ± SD of three independent experiments. E. Effects of DPI on PMA-induced neutrophil superoxide generation. Neutrophils were treated or not with 10 μM DPI for 30 min at 37°C. The loaded cells were then incubated with 10 nM PMA for 30 min at 37°C. Each bar shows the mean ± SD of three independent experiments. F. Effects of DPI on anti-PtdGlc Ab-induced neutrophil apoptosis. Neutrophils were treated or not with 10 μM DPI for 30 min at 37°C. The loaded cells were then incubated with 4 μg/ml anti-PtdGlc Ab-DIM21 for 4 h at 37°C. Each bar shows the mean ± SD of three independent experiments. **p < 0.01, ***p < 0.001.
them useful as differentiation markers. Although several types of
glycolipid are present on neutrophils (53), the functions of most
of these molecules, with the exception of LacCer, have not been
well characterized. LacCer is a differentiation marker for human
neutrophils (54, 55), but it is also expressed on monocytes (54)
and monocyte-derived DCs (Fig. 1). In contrast, PtdGlc was
weakly expressed on monocytes and lymphocytes, and it was not
expressed on monocyte-derived DCs. Therefore, it is likely that
PtdGlc, rather than LacCer, can be used as a highly specific dif-
ferentiation marker for neutrophils.

Previous studies demonstrated the presence of heterogeneous
lipid domains on plasma membranes in the same cells (56, 57).
PtdGlc forms distinct lipid domains from glycosphingolipids on
the outer leaflet of the plasma membrane of HL60 cells and the
human alveolar epithelial cell line A549 (18). Confocal micro-
scopic examination of neutrophils revealed that PtdGlc was not
colocalized with LacCer on the plasma membrane (Fig. 3). LacCer
mediates neutrophil chemotaxis and superoxide generation
but not apoptosis (5, 8). In contrast, PtdGlc mediates
neutrophil apoptosis but not chemotaxis or superoxide generation
(Figs. 3, 4). LacCer-mediated signal transduction is highly de-
pendent on the activation of Lyn and PIP3 kinase (6, 58), whereas
PtdGlc-mediated neutrophil apoptosis did not depend on Src
family kinases or PIP3 kinase (Fig. 6). Several studies demon-

FIGURE 7. Association of PtdGlc and Fas. A, Colocalization of Fas and PtdGlc. Neutrophils (2 × 10^6 cells/ml) were incubated without (Resting) or with
5 μg/ml anti-Fas IgDGD2, anti-PtdGlc IgM DIM21, or anti-sialyl Le^a IgM CSLEX1 at 37°C for 5 min. The cells were washed with 10 volumes of ice-cold
PBS, fixed with PLP, and treated sequentially with primary DX2 or DIM21 Abs and secondary (Alexa Fluor 488-conjugated goat anti-mouse IgG or Alexa
Fluor 647-conjugated rabbit anti-mouse IgM) Abs for 30 min on ice. The stained cells were examined with a TCS-S2 Leica confocal microscope equipped
with a Plan-Apochromat 100× oil-immersion DIC objective. Scale bars, 10 μm. B, Fluorescence intensity profiles of plasma membrane area in image
overlays shown in A. C, Cytofluorogram from Alexa Fluor 488-conjugated anti-Fas IgG (Fas) and Alexa Fluor 546-conjugated anti-PtdGlc DIM21 (PtdGlc)
in image overlays shown in A. The numbers indicate the mask intensity rates. D, Neutrophils were treated without (−) or with 10 μg/ml anti-Fas IgG ZB4
for 30 min on ice. After incubation, cells were washed and stained with Alexa Fluor 488-DIM21. BG. Cells were treated with normal IgM on ice and
stained with Alexa Fluor 488-labeled anti-IgM. E, Effects of the Fas antagonist Ab ZB4 on PtdGlc-mediated neutrophil apoptosis. Neutrophils (2.5 × 10^6
cells/ml) were incubated or not without 10 μg/ml anti-Fas IgG ZB4 on ice for 60 min, followed by the addition of 2, 4, or 8 μg/ml anti-PtdGlc IgM DIM21,
4 μg/ml anti-Fas IgM CH-11(CH-11), or 4 μg/ml normal IgM and incubation at 37°C for 4 h. The binding of Annexin V to the cells was analyzed as
described in Materials and Methods. ●, DIM21; ○, DIM21+ZB4. **p < 0.01, ***p < 0.001.
strated that Src family kinases, including Lyn, suppress apoptosis by inhibiting caspase-8 activity (34). Many factors that promote neutrophil survival activate PIP2 kinase (59). Therefore, it seems that LacCer and PtdGlc form different domains on the neutrophil plasma membrane and mediate different functions via different molecular mechanisms.

Apoptosis can be initiated by two main pathways: the death receptor-mediated pathway and the intrinsic mitochondrial pathway (60). The Fas-mediated pathway belongs to the death receptor pathway. Ligation of Fas induces the formation of a death-inducing signaling complex consisting of Fas, an adaptor protein (FADD) for Fas, and procaspase-8 (60). Fas and its ligand exist as trimers, and the clustering of Fas-containing domains following ligation is required to promote aggregation of procaspase-8 molecules within the death-inducing signaling complex (48). The coalescence of Fas-containing lipid rafts after ligation of this receptor is the initial step of Fas-induced apoptosis (61). Consistent with these results, we observed that cross-linking of Fas caused the formation of a large cluster of Fas-containing domains, which contained PtdGlc molecules (Fig. 7). Importantly, the cross-linking of PtdGlc also formed Fas-containing large clusters on the plasma membrane of neutrophils. Moreover, the Fas antigenic Ab ZB4 significantly blocks apoptosis induced by CH-11 through binding to the same epitope as CH-11 (50). In contrast, the antigenic anti-Fas IgG ZB4, which has the ability to prevent Fas ligation (51), significantly inhibited PtdGlc-mediated neutrophil apoptosis (Fig. 7E). Therefore, it is likely that the Fas-mediated pathway is responsible for PtdGlc-mediated neutrophil apoptosis.

Identification of the natural ligands for PtdGlc in vivo is the most important issue involving PtdGlc-mediated neutrophil apoptosis that remains to be solved. PtdGlc-enriched raft-like domains express glucose clusters on the neutrophil plasma membrane. Glucose residues can be recognized by certain kinds of glucose-binding lectin, such as glucose/mannose-specific lectins and C-type animal lectins. Indeed, macrophage mannose receptors, which are expressed on human macrophages and lymphocytes, are capable of binding to glucose residues (62, 63). Apoptosis is the final step in the differentiation of neutrophils. Apoptosis is necessary to maintain a certain number of neutrophils in vivo and for macrophages to remove neutrophils from inflamed tissues, steps critical in limiting inflammatory tissue injury and the subsequent resolution of inflammation (2–4, 64). Apoptosis of neutrophils may be responsible for neutrophil apoptosis that remains to be resolved. PtdGlc-enriched raft-like domains are required for CD11b/CD18-mediated neutrophil phagocytosis of nonopsonized microorganisms. J. Leukoc. Biol. 83: 728–741.


