Glycosylation Failure Extends to Glycoproteins in Gestational Diabetes Mellitus

Evidence From Reduced α2-6 Sialylation and Impaired Immunomodulatory Activities of Pregnancy-Related Glycodelin-A

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OBJECTIVE—Gestational diabetes mellitus (GDM) is a common metabolic disorder of pregnancy. Patients with GDM are at risk for high fetal mortality and gestational complications associated with reduced immune tolerance and abnormal carbohydrate metabolism. Glycodelin-A (GdA) is an abundant decidual glycoprotein with glycosylation-dependent immunomodulatory activities. We hypothesized that aberrant carbohydrate metabolism in GDM was associated with changes in glycosylation of GdA, leading to defective immunomodulatory activities.

RESEARCH DESIGN AND METHODS—GdA in the amniotic fluid from women with normal (NGdA) and GDM (DGdA) pregnancies was purified by affinity chromatography. Structural analysis of protein glycosylation was preformed by lectin-binding assay and mass spectrometry. Cytotoxicity, cell death, cytokine secretion, and GdA binding of the GdA-treated lymphocytes and natural killer (NK) cells were determined. The sialidase activity in the placental tissue from normal and GDM patients was measured.

RESULTS—GDM affected the glycosylation but not the protein core of GdA. Specifically, DGdA had a lower abundance of α2-6–sialylated and high-mannose glycans and a higher abundance of glycans with Sda (NeuAcα2-3[GalNAcβ1-4]Gal) epitopes compared with NGdA. DGdA had reduced immuosuppressive activities in terms of cytotoxicity on lymphocytes, inhibitory activities on interleukin (IL)-2 secretion by lymphocytes, stimulatory activities on IL-6 secretion by NK cells, and binding to these cells. Desialylation abolished the immunomodulation and binding of NGdA. Placental sialidase activity was increased in GDM patients, which may account for the reduced sialic acid content of DGdA.

CONCLUSIONS—Taken together, this study provides the first direct evidence for altered enzymatic glycosylation and impaired bioactivity of GdA in GDM patients. ***Diabetes*** **60:909–917, 2011**

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Gestational diabetes mellitus (GDM) is a metabolic disorder manifested as glucose intolerance with an onset during pregnancy (1,2). It occurs in ~7% of pregnancies in the U.S. (1) and in 14% of pregnancies in Hong Kong (3). Compared with normal pregnancy, GDM is associated with a higher risk of pregnancy complications such as macrosomia, preeclampsia, fetal mortality, placental alterations, and increased risk of diabetes of the mother and the offspring later in life (2,4,5). The underlying pathophysiology of GDM has been associated with dysregulated immune responses, as demonstrated by changes in the immune cell subpopulations and the cytokine profile in women with GDM (6,7).

Glycodelin-A (GdA) is an abundant secretory glycoprotein of the pregnancy decidua (8). It is proposed to be involved in fetomaternal defense (9,10) through its immunomodulatory activities. These include induction of apoptosis of T-cells (11), skewing of T-cell response toward the Th-2 phenotype (12), and modulation of the activities of natural killer (NK) cells (13), B-cells (14), and dendritic cells (15). Decreased secretion of glycodelin is associated with recurrent spontaneous abortion and unexplained infertility (8,10).

Glycosylation is crucial to the biological activities of glycodelins (8,10). Its importance is shown by the lack of immunomodulatory activity in two other glycodelin isoforms with different glycosylation, glycodelin-S and glycodelin-C (11). Although there is no difference in the glycodelin concentration of the maternal serum in the first trimester (16) and of the cord serum (17) between GDM and normal pregnancy, the glycosylation of glycodelin in GDM pregnancies is unknown. Hyperglycemia in diabetes causes abnormal carbohydrate metabolism and the production of advanced glycation end products, leading to alteration of gene expression and activities of the cellular glycosyltransferases and glycosidases (18). Diabetic pregnancy has been associated with changes in the glycosylation and subsequently the biological activities of human chorionic gonadotrophin (19) and the placental transferrin receptor (20). However, because of the lack of advanced methodological procedures, these studies did not provide any detailed information of the glycan structures and the resultant changes in biological activities.

We hypothesized that the aberrant carbohydrate metabolism in GDM was associated with alteration in glycosylation of GdA, thereby leading to defective immunomodulatory activities of the molecule during pregnancy. To test this hypothesis, we compared the immunomodulatory activities of GdA from normal (NGdA) and GDM (DGdA) pregnancy and determined the changes in their *N*-glycan structures by mass spectrometric analysis. The results showed differences in glycosylation between NGdA and DGdA. In particular, DGdA had reduced sialylation, leading to reduced binding to lympohocytes and therefore decreased immunomodulatory activity of the molecule. The results support the hypothesis that GDM-associated changes in glycosylation alter the biological activities of GdA.

RESEARCH DESIGN AND METHODS

Normal and diabetic amniotic fluid samples. The study protocol was approved by the institutional review board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. A total of 35 amniotic fluid samples (20 normal and 15 GDM) were collected from women at term pregnancy during cesarean delivery at the Queen Mary Hospital, Hong Kong. The diagnosis of GDM was according to the World Health Organization criteria using a 2-h 75-g oral glucose tolerance test (OGTT), as described (3). Blood glucose levels >7.8 mmol/L were defined as having GDM. Two women with GDM were treated by insulin and 13 women with GDM were treated through diet control. Maternal and infant demographic information of normal and GDM participants are shown in Table 1. The two groups of women were similar in gravidity, parity, age, BMI, gestational age at birth, fasting plasma glucose, and placental weight and fetal birth weight and differed only in 2-h plasma glucose during the OGTT. The amount of the GdA isolated from each amniotic fluid sample was limited. Therefore, NGdA and DGdA were randomly pooled into five groups for experimentation.

Cell culture. Human female peripheral blood was obtained from the Hong Kong Red Cross. Peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll-Paque PLUS method (GE Healthcare, Uppsala, Sweden). Peripheral blood NK (pbNK) cells with a purity of >95% were isolated from PBMCs using a negative isolation kit (Dynal Biotech, Oslo, Norway). These cells were cultured in 10% FBS supplemented with RPMI 1640 medium containing 500 IU/mL recombinant interleukin (IL)-2 (Sigma-Aldrich, St. Louis, MO).

PMBCs, Jurkat (T-lymphoma cells), and OE-E6/E7 (oviductal cell line) were cultured in RPMI-1640 medium (Sigma-Aldrich). TEV-1 (trophoblast cell line) was cultured in DMEM medium (Sigma-Aldrich). All the culture media were supplemented with 10% FBS.

Determination of glycodelin concentration in amniotic fluid. The glycodelin content from the amniotic fluid of normal and GDM pregnancies was assayed by enzyme-linked immunosorbent assay (ELISA). Polyclonal goat anti-human glycodelin antibody (1 μg in 100 μL; R&D Systems, Minneapolis, MN)-coated assay wells were incubated successively with the amniotic fluid sample or GdA standard (0–10 μg/mL), murine monoclonal antiglycodelin antibody (clone F43-7F9), and horseradish peroxidase–conjugated goat anti-mouse IgG (1:5000 vol/vol; Sigma-Aldrich). The ELISA signal was determined by absorption at 450 nm using a microplate reader (MR5000; Dynatech Laboratories, Chantilly, VA). GdA content was expressed as a ratio to the total protein content of the amniotic fluid.

Purification of GdA. NGdA and DGdA were purified from amniotic fluid as described (21). Desialylation of NGdA was performed using sialidase-coated agarose beads (Sigma-Aldrich) at 37°C for 18 h. The success of desialylation was verified by the decreased binding to wheat germ agglutinin lectin (11). The concentration of purified GdA was estimated by a protein assay kit (Bio-Rad, Hercules, CA). The purity of the isolated GdA was checked by 12% SDS-PAGE, whereas the identity of the isolated GdA was confirmed by peptide mass fingerprinting of the GdA band in SDS-PAGE using matrix-assisted laser desorption/ionization–tandem time-of-flight mass spectrometer (4800 MALDI-TOF/TOF; Applied Biosystems, Warrington, U.K.).

Lectin binding assay. Glycosylation of glycodelins was studied by a lectin-binding assay as described (11). Briefly, assay wells coated with various lectins (7.5 pmol per well) were blocked by 5% casein before successive incubation with GdA (250 ng per well), monoclonal antiglycodelin antibody (1 μg per well), and horseradish peroxidase–conjugated anti-mouse IgG (1:5000 vol/vol; Sigma-Aldrich). The signal was developed with *o*-phenylenediamine (Sigma-Aldrich) and determined as described above.

Glycan sequencing by MALDI-TOF and MALDI-TOF/TOF. Purified NGdA and DGdA (~15 μg each) were digested using trypsin (Sigma-Aldrich) and purified by a reverse-phase Sep-Pak C18 cartridge (Waters Corporation, Manchester, U.K.) as described (22). The *N*-glycans were then released by *N*-glycosidase F (Roche Applied Science, West Sussex, U.K.) and purified on a Sep-Pak C18 cartridge. The purified native *N*-glycans were permethylated as described (23), purified using a Sep-Pak C18 cartridge, dissolved in methanol, and mixed with 20 mg/mL 2,5-dihydrobenzoic acid in 70% methanol at a 1:1 ratio (vol/vol). The glycan-matrix mixture (1 μL) was spotted on a stainless-steel target plate and dried in a vacuum. MALDI-TOF and -TOF/TOF data were obtained using a 4800 MALDI-TOF/TOF mass spectrometer (AB Sciex UK Limited, Warrington, U.K.). Argon with a collision energy of 1 kV was used. The mass spectrometry (MS) and tandem MS (MS/MS) data obtained were analyzed using Data Explorer 4.9. The assignment of glycan sequence was done by manual annotation informed by knowledge of human biosynthetic pathways and aided by the glycobioinformatics tool, GlycoWorkBench (24). Relative quantification of the glycan abundance in a single spectrum was calculated relative to the total ion counts from all of the observed glycans (percentage of total abundance = [ion count of the glycan/total ion count of all glycans] × 100%).

Linkage analysis by gas chromatography–mass spectrometry. The *N*-glycans were analyzed by gas chromatography–mass spectrometry (GC-MS) as described (23). The samples were dissolved in hexane before injection into the gas chromatographer–mass spectrometer (Clarus 500; PerkinElmer, Waltham, MA) fitted with an RTX-5 column (30 m × 0.32 mm internal diameter; Restek, Bellefonte, PA). The oven temperature was held at 90°C for 1 min and subsequently ramped to 290°C at a rate of 8°C per min. The acquired data were analyzed by TurboMass version 4.5.0.007 (Perkin Elmer Instruments, Shelton, CT).

TABLE 1
Maternal and infant demographic information of normal and GDM participants

Demographic information	Normal (n = 20)	GDM (n = 15)
Gravidity	2.8 (1–5) ± 1.25	2.4 (1–5) ± 1.33
Parity	1.8 (1–3) ± 0.62	1.7 (1–4) ± 0.95
Maternal age (years)	35.0 (28–43) ± 3.40	35.6 (29–40) ± 3.36
Maternal BMI (kg/m^2)	27.1 (22.2–31.8) ± 2.55	27.5 (20.9–38.6) ± 5.22
Gestational age at birth (weeks)	38.8 (37.6–41.1) ± 1.06	38.3 (37.6–40.9) ± 0.90
Fasting plasma glucose (mmol/L)	4.4 (3.5–5.3) ± 0.50	4.8 (3.8–7.4) ± 0.96
2-h plasma glucose (mmol/L)	6.1 (5.2–7.6) ± 0.57	9.4 (7.8–11.6) ± 1.18*
Placental weight (g)	559.5 (430–740) ± 88.9	553.6 (460–700) ± 87.9
Fetal birth weight (g)	3,178.5 (2,165–4,295) ± 460.7	3,108.1 (2,570–3,755) ± 396.7
Indications for caesarean section	Normal (n = 20) Pervious caesarean section scar × 16 Fetal malpresentation × 2 High head × 2	GDM (n = 15) Pervious caesarean section scar × 8 Fetal malpresentation × 1 High head × 1 Cervical incompetence × 2 Cephalopelvic disproportion × 3

Data are means (range) ± SD. *P < 0.001 vs. normal participants.

XTT cell viability assay. Cell viability was determined by the XTT assay (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. The absorbance of the resulting color product was measured at 450 nm with a λ correction at 595 nm. The changes in cell viability were expressed as the suppression index (S_I) using the following equation: suppression index (%) = (Abs GdA − Abs blank) × 100%.

Cell death analysis by flow cytometry. Apoptotic and necrotic cell death were determined by flow cytometry using Yo-Pro-1 and propidium iodide dye (Invitrogen, Carlsbad, CA) and analyzed with a flow cytometer (Beckman Coulter, Fullerton, CA) equipped with a 488-nm argon laser. The fluorescence signal was measured using the 525-nm and 610-nm band pass filters and was analyzed by the Winlist software (Verity Software House, Topsham, ME).

Determination of cytokine production by ELISA. The levels of IL-2 and IL-6 in the conditioned media were measured by ELISA (IL-2, BD Biosciences, Franklin Lakes, NJ; IL-6, Invitrogen, Carlsbad, CA), according to the manufacturer's procedure. The absorbance derived from 3.3′,5.5′-tetramethylbenzidine was measured at 450 nm with a λ correction of 595 nm as above.

Glycodelin binding assay. The binding of GdA was visualized by flow cytometric analysis. In brief, GdA was fluorescence labeled using the Alexa Fluor 488 microscale fluorescence labeling kit (Invitrogen) as described (25). The cells (5×10^5) were incubated with 1 μg of Alexa Fluor 488–labeled GdA for 2 h, washed with PBS twice, and analyzed by flow cytometry as above.

Determination of placental sialidase activity. Human placentae were obtained from 20 singleton pregnancies (10 normal pregnancies and 10 GDM pregnancies) after elective cesarean section at term before the onset of labor at the Queen Mary Hospital, Hong Kong. The tissues were dissected, washed with PBS, and homogenized in the presence of protease inhibitors and phosphatase inhibitor. The sialidase activity in the total cell lysates (50 μg) was determined by a fluorimetric assay using an artificial substrate, 4-methylumbelliferyl *N*-acetylneuraminic acid (Sigma-Aldrich) as described (11). Fluorescence emission was measured by a fluorometer with excitation at 340 nm and emission at 505 nm (Infinite F200; Tecan, Männedorf, Switzerland).

Data analysis. All values were expressed as means ± SEM. The data were compared by ANOVA to discern differences between groups. Parametric Student *t* test or a nonparametric Mann-Whitney *U* test were used where appropriate as the posttest. A *P* value <0.05 was considered significant.

RESULTS

Purification and identification of NGdA and DGdA. There was no significant difference in the amount of GdA in the amniotic fluid from normal (0.90 ± 0.32 μg/mg total protein; $n = 20$) and GDM (0.71 ± 0.56 μg/mg; $n = 15$) pregnancies. Purified NGdA and DGdA had a similar molecular size in SDS-PAGE (~30 kDa, Supplementary Fig. 1) and peptide mass fingerprinting in MS/MS (Supplementary Fig. 1). The peptide mass fingerprints of both NGdA and DGdA were significantly matched to the product of the progesterone-associated endometrial protein gene (protein score = 162 for NGdA, $P < 0.001$; protein score = 120 for DGdA, $P < 0.001$), a gene-encoding glycodelin.

NGdA and DGdA have different lectin-binding affinities. DGdA reacted weakly to concanavalin A (ConA), suggesting a low abundance of mannose/glucose in its glycans. DGdA also had reduced affinity to sialic acid (*N*-acetyl-5-neuraminic acid) and *N*-acetylglucosamine (GlcNAc)-binding lectin and wheat germ agglutinin (WGA) (Table 2). Because NGdA and DGdA had similar affinity to succinylated wheat germ agglutinin (*S*-WGA), a lectin that binds to *N*-acetylglucosamine, the reduced binding affinity of DGdA to WGA reflected a lower amount of sialylated glycans in the molecule.

Differential glycomics between NGdA and DGdA. Glycomics analysis was performed using strategies previously optimized for GdA characterization (11). The permethylated *N*-glycans were subjected to MALDI-MS profiling and MALDI-MS/MS sequencing. Linkage analysis using GC-MS was subsequently carried out on the remaining samples. The complete MALDI spectra of NGdA and DGdA glycans are shown in single panels in Fig. 1 to facilitate visual comparison. For clarity, only the most informative molecular ions are annotated with glycan structures in this figure. Comprehensive annotations are shown on the magnified spectra, which are reproduced in Supplementary Fig. 2. Because the amounts of purified NGdA and DGdA were limited, only strong signals in the spectra could be sequenced by MALDI-TOF/TOF MS/MS. These components are flagged in Supplementary Fig. 2. Combining information on the glycan compositions, structure, and linkage, the structures were assigned manually, based on knowledge of human *N*-glycan biosynthetic pathways.

TABLE 2
Binding of lectins with NGdA and DGdA

Lectin (specificity)	Lectin immunoassay at OD_{450}	
	NGdA	DGdA
Wisteria floribunda agglutinin (GalNAc)	0.55 ± 0.09	0.53 ± 0.02
Sambucus nigra bark agglutinin (−NeuNAc[2-6]Gal/GalNAc)	0.63 ± 0.03	0.58 ± 0.11
Concanavalin A (ConA) (−Man, −Glc)	0.59 ± 0.04	0.42 ± 0.05*
WGA ($[GlcNAc]_2$, NeuNAc)	0.42 ± 0.05	0.04 ± 0.02*
S-WGA (GlcNAc or its oligomer)	0.11 ± 0.05	0.17 ± 0.04

Data are means ± SEM, $n = 5$. *$P < 0.05$ vs. NGdA at the same concentration.

The *N*-glycans were highly complex, and most of the abundant glycans were biantennary and triantennary glycans. Some mass peaks of the same *m/z* value contained mixtures of glycans (e.g., glycans of *m/z* of 1,662, 1,836, 1,866, 2,070, 2,244, 2,285, 2,489, 2,693, 2,717, 2,850, and 3,253). The glycans of both NGdA and DGdA comprised high-mannose, hybrid, and abundant complex structures. NGdA from term pregnancy shared the same glycosylation pattern as that reported for GdA from midtrimester pregnancy (11); both had heavily sialylated glycans, Lewis X glycans, the Sda (NeuAcα2-3[GalNAcβ1-4]Gal) epitope in the high–molecular weight glycans and bisecting *N*-acetylglucosamine on some of the biantennary glycans. Some common characteristics of *N*-glycans in many glycoproteins were observed, such as lacNAc and lacdiNAc as antenna backbones, sialylated lacNAc or lacdiNAc antennae, fucosylated lacNAc or lacdiNAc, and fucosylated core GlcNAc. Linkage analysis by GC-MS (Supplementary Table 1) showed that DGdA contained terminal fucose, mannose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine, 2-linked mannose, 6-linked galactose, 3,4-linked galactose, 2,4-linked mannose, 3,6-linked mannose, 3,4,6-linked mannose, 4-linked GlcNAc, and 4,6-linked GlcNAc. In addition, linkage analysis gave evidence for the specific glycan structures of GdA, such as Sda (3,4-linked galactose), bisecting GlcNAc (3,4,6-linked mannose), core fucose (4,6-linked GlcNAc), and terminal sialic acid (6-linked galactose). Comparison of these DGdA linkage data with published linkage data for NGdA (11) reveals substantially lower levels of 6-linked galactose and 6-linked *N*-acetylgalactosamine in the former compared with the latter. This is indicative of lower levels of α2-6 sialylation in DGdA compared with NGdA.

The relative abundances of the glycans of NGdA and DGdA were compared (Fig. 1). Some important differences were found: *1*) Of 147 glycoforms identified, >55% were

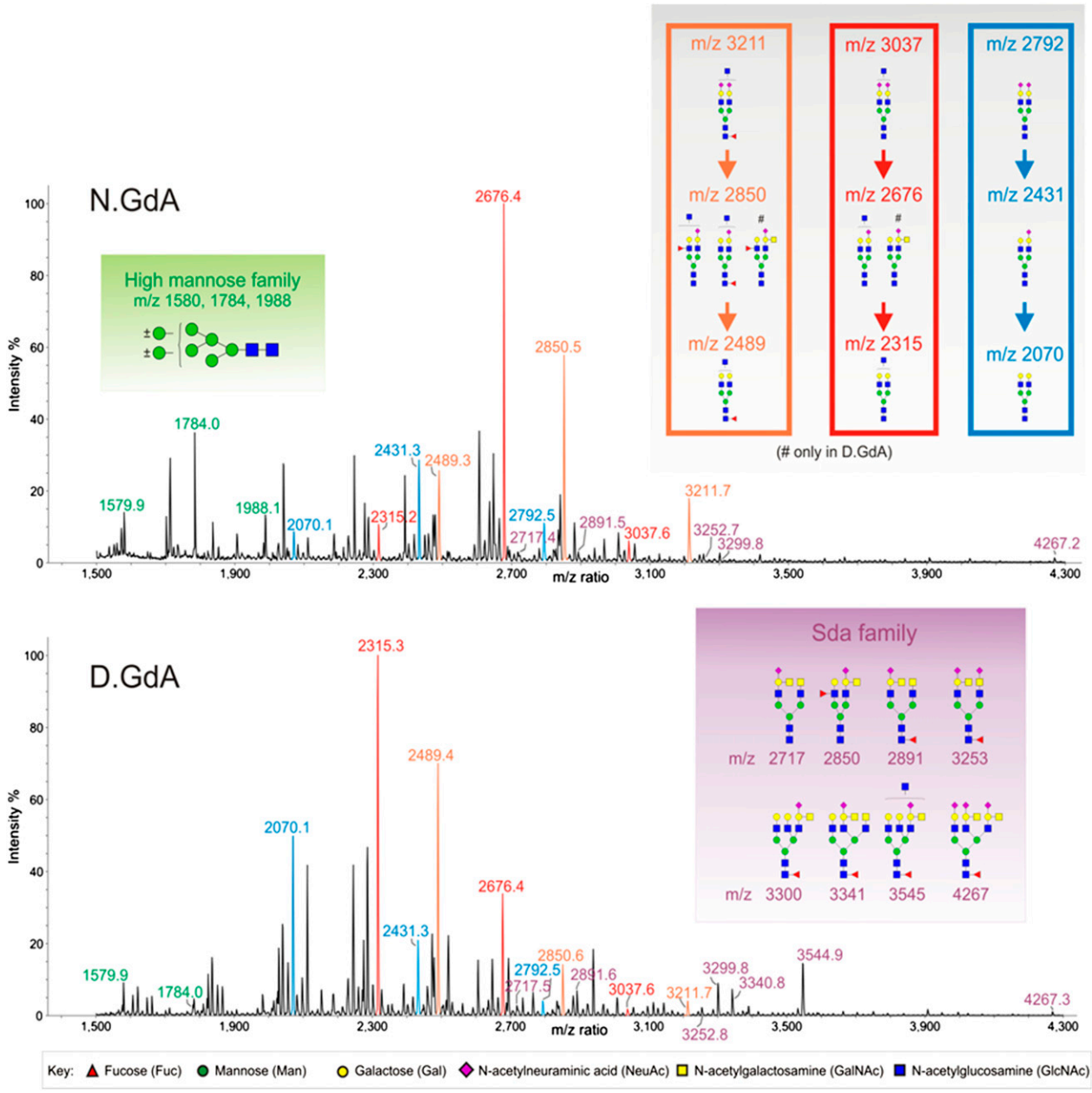


FIG. 1. MALDI-TOF mass spectra of *N*-glycans of NGdA and DGdA (m/z 1500–4000). The *N*-glycans from purified glycodelin preparations were released by PNGase F and permethylated (RESEARCH DESIGN AND METHODS) prior to MALDI-TOF profiling. For ease of semiquantitative comparison, each spectrum is shown in a single panel, and all data are normalized to the most abundant component, which is designated as 100%. For clarity, not all molecular ions are annotated with their m/z values. The annotated signals exemplify the major differences between the NGdA and DGdA glycomes. Color coding has been used to distinguish families of glycans. The α2-6 sialylated glycans and their desialylated counterparts are flagged as orange, red, and blue peaks in the spectra, and their annotations are shown in the respective *orange*, *red*, and *blue panels*. Thus, orange indicates glycans that are bisected and fucosylated, those in red are bisected but not fucosylated, and those in blue are neither bisected nor fucosylated. The upper structures in each of these panels are fully sialylated, and the arrows depict loss of sialic acid. The *green* and *magenta inserts* show high-mannose glycans and the Sda family of complex glycans, respectively. Fully annotated spectra, which have been expanded on the m/z axis to enable all components to be visualized, are presented in Supplementary Fig. 2. (A high-quality color representation of this figure is available in the online issue.)

found in both NGdA and DGdA (Supplementary Fig. 2). *2*) The most notable difference between the two samples was that DGdA contained much lower levels of sialylated glycans (DGdA: 24.7%; N.GdA: 53.4%). From the MALDI-MS spectra, many of the strong peaks of NGdA shifted toward low molecular weight by 361 mass units in DGdA, corresponding to the molecular weight of permethylated sialic acid, indicating that these glycans lacked sialic acid in DGdA (see the *orange*, *red*, and *blue panels* in Fig. 1). *3*) DGdA contained a lower abundance (1.9 vs. 10.3% of NGdA) of high-mannose glycans (*m/z* 1,580, 1,784, and 1,988). This is consistent with the reduced ConA binding affinity of DGdA in the lectin-binding assay. The low abundance of high-mannose glycans suggested that they were unlikely to occupy a glycosylation site fully, as previously described for the Asn28 site of GdS, which is exclusively occupied by high-mannose glycans (10). *4*) DGdA contained more glycans capped with the Sda epitope

(e.g., *m/z* 2,717, 2,850, 2,891, 3,253, 3,300, 3,341, 3,545, and 4,267; see the *magenta panel* in Fig. 1), which was a recently identified characteristic of the female glycodelins (11). The smaller amount of sialylated glycans in DGdA compared with NGdA was further confirmed by the decreased WGA-binding of DGdA in a second population of normal and GDM patients (Supplementary Table 2).

Reduced cytotoxicity of DGdA and desialylated NGdA on human lymphocytes. Treatment with both NGdA and DGdA at concentrations of ≥0.01 μg/mL for 36 h significantly ($P < 0.05$) decreased the viability of PBMCs, the cytotoxicity of the latter was significantly ($P < 0.05$) lower than that of the former at concentrations of 0.01 and 0.1 μg/mL (Table 3). NGdA, but not DGdA, at a concentration of 0.1 μg/mL, significantly ($P < 0.05$) decreased the viability of Jurkat cells. At 1 μg/mL, the cytotoxic effect of NGdA on Jurkat cells was also significantly ($P < 0.05$) higher than that of DGdA (viability: NGdA, 41.7 ± 4.9%; DGdA, 79.4 ± 8.2%). At the tested concentrations, neither NGdA nor DGdA affected the viability of TEV-1 and OE-E6/E7 cells.

Compared with NGdA, the cytotoxic effect of desialylated NGdA could only be observed at higher concentrations (PBMCs: ≥0.1 μg/mL; Jurkat cells: ≥1 μg/mL). In addition, the suppression index of desialylated NGdA was higher than that of NGdA when tested at the same concentrations.

DGdA and desialylated NGdA have impaired ability to induce cell death of lymphocytes. Treatment with 0.1 μg/mL NGdA significantly ($P < 0.01$) decreased the viable population of PBMCs from 87.0 ± 5.7% to 26.9 ± 2.9% in the YoPro-PI assay (Table 4 and Supplementary Fig. 3). The corresponding decrease by DGdA was significantly ($P < 0.05$) smaller (from 87.0 ± 5.7% to 42.4 ± 15.5%), indicating a lower cytotoxic activity of DGdA on PBMCs.

Treatment with 0.1 μg/mL NGdA for 48 h significantly ($P < 0.05$) decreased the percentage of viable Jurkat cells from 86.6 ± 2.4% to 77.2 ± 3.5% (Table 4 and Supplementary Fig. 3). By contrast, DGdA at the same concentration had no significant effect on the viability of these cells (82.9 ± 1.8%). A differential response ($P < 0.05$) of Jurkat cells to NGdA and DGdA was also observed at the concentration of 1 μg/mL.

After desialylation, the ability of NGdA to induce cell death of PBMCs and Jurkat cells was abolished (Table 4 and Supplementary Fig. 3). No significant difference was observed on the viable population after treatment with desialylated NGdA when compared with the control.

DGdA and desialylated NGdA have reduced ability in modulating the cytokine secretion by lymphocytes and NK cells. NGdA dose-dependently inhibited IL-2 secretion by PBMCs and Jurkat cells (Table 5). DGdA and desialylated NGdA had a significantly ($P < 0.05$) lower suppressive effect on IL-2 secretion than that of NGdA at the same concentration in either cell types. Neither NGdA nor DGdA affected cell viability within the treatment period (data not shown). For IL-6, DGdA and desialylated NGdA had a significantly ($P < 0.05$) smaller stimulatory effect on pbNKs when compared with NGdA at the concentration of 1 μg/mL (Table 5).

DGdA and desialylated NGdA have reduced binding affinity to the lymphocytes. The binding affinity of DGdA on Jurkat and pbNK cells was significantly lower ($P < 0.05$) than that of NGdA (Fig. 2). The binding of DGdA on PBMCs was also somewhat lower, though the difference did not reach statistical significance (NGdA: 35.7 ± 5.3%; DGdA: 27.6 ± 6.0%). Upon desialylation, the binding of desialylated NGdA became significantly reduced in all the cells tested.

Placental tissue of GDM has a higher sialidase activity. The sialidase activity of the GDM placental tissue increased the production of the 4-methylumbelliferone in a time-dependent manner and was significantly ($P < 0.05$) higher than that of the normal placental tissue (Fig. 3).

DISCUSSION

Changes in glycosylation of glycoproteins occur in the normal menstrual cycle and during pregnancy (26,27).

TABLE 3
Effect of NGdA, DGdA, and desialylated NGdA on viability of PBMCs, Jurkat cells, TEV-1, and OE-E6/E7 by XTT assay

	Suppression index (S_I ± SEM)			
	PBMCs	Jurkat cells	TEV-1	OE-E6/E7
NGdA				
GdA (μg/mL)				
0.001	95.0 ± 1.7	104.7 ± 1.7	103.8 ± 3.5	100.4 ± 1.6
0.01	67.9 ± 1.4*	93.0 ± 8.3	103.5 ± 3.0	102.4 ± 1.2
0.1	40.1 ± 0.8*	68.6 ± 2.8*	101.0 ± 2.2	101.1 ± 1.5
1	37.1 ± 0.5*	41.7 ± 4.9*	98.8 ± 6.7	97.9 ± 1.5
DGdA				
GdA (μg/mL)				
0.001	97.6 ± 8.7	100.5 ± 1.3	105.8 ± 2.7	101.3 ± 1.1
0.01	83.7 ± 3.8*†	96.6 ± 3.8	105.5 ± 1.6	102.1 ± 1.4
0.1	74.5 ± 3.8*†	102.7 ± 2.4†	103.1 ± 1.6	100.4 ± 1.6
1	42.6 ± 2.0*	79.4 ± 8.2*†	94.2 ± 10.8	97.3 ± 1.9
Desialylated NGdA				
GdA (μg/mL)				
0.001	100.1 ± 4.1	100.5 ± 1.4	—	—
0.01	100.3 ± 4.0†	100.8 ± 1.4	—	—
0.1	81.6 ± 11.6*†	98.7 ± 1.9†	—	—
1	53.1 ± 10.0*	89.8 ± 3.7*†	—	—

Data are mean ± SEM, $n = 5$. Cells (3 × 104) were incubated with 0.001, 0.01, 0.1, and 1 μg/mL glycodelin for 36 h, and XTT-labeling mixture was added 12 h before measurement. Suppression index (%) = (Abs GdA − Abs blank) / (Abs control − Abs blank) × 100%. *$P < 0.05$ vs. control without treatment. †$P < 0.05$ vs. NGdA at the same concentration.

TABLE 4
Effect of NGdA, DGdA, and desialylated NGdA on the cell death of PBMCs and Jurkat cells

	Control	NGdA (μg/mL)			DGdA (μg/mL)			Desialylated NGdA (μg/mL)		
		0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
PBMCs (%)										
Viable	87.0 ± 5.7	44.3 ± 15.4*	26.9 ± 2.9*	20.8 ± 3.2*	57.1 ± 1.3*†	42.4 ± 15.5*†	23.7 ± 5.4*	76.7 ± 7.7	75.7 ± 8.4†	71.3 ± 8.3†
Necrosis	8.0 ± 4.0	42.5 ± 13.7*	59.9 ± 1.8*	64.7 ± 3.4*	25.7 ± 1.4*	41.2 ± 16.2*	62.0 ± 5.5*	15.0 ± 4.7†	15.8 ± 5.6†	19.6 ± 5.7†
Apoptosis	4.8 ± 1.3	12.8 ± 1.8*	12.9 ± 1.3*	14.1 ± 0.6*	16.5 ± 0.3*†	15.8 ± 1.6*	13.8 ± 1.2*	7.4 ± 2.7†	7.6 ± 2.7†	8.1 ± 2.5†
Jurkat cells (%)										
Viable	86.6 ± 2.4	77.0 ± 3.3*	77.2 ± 3.5*	61.9 ± 11.8*	82.0 ± 2.6	82.9 ± 1.8†	77.0 ± 4.1*†	83.9 ± 1.5	83.0 ± 1.4†	83.9 ± 1.3†
Necrosis	6.6 ± 1.8	13.2 ± 2.2*	16.7 ± 2.8*	34.9 ± 11.5*	10.2 ± 0.9*	9.2 ± 0.7	15.8 ± 2.4*†	9.7 ± 1.2*	10.1 ± 0.5*	9.0 ± 1.2†
Apoptosis	4.2 ± 1.5	8.7 ± 1.2*	5.5 ± 0.8	2.7 ± 0.7	7.1 ± 1.8	7.2 ± 1.2*	6.6 ± 1.9†	6.3 ± 0.4	6.8 ± 1.1	7.0 ± 1.0*†

Data are means ± SEM, $n = 5$. PBMC and Jurkat cells (5×10^5) were incubated with 0–1 μg/mL glycodelins for 48 h. Viable, necrotic, and apoptotic cells were identified and quantified by bivariate Yo-Pro-1/PI flow cytometry. Cells without stain were counted as viable cells. Cells labeled with Yo-Pro-1 only were counted as apoptotic cells. Cells labeled with both Yo-Pro-1 and PI was counted as necrotic cells. *$P < 0.05$ vs. control without treatment. †$P < 0.05$ vs. NGdA at the same concentration.

Altered glycosylation of glycoproteins and glycolipids occur in diabetes, cancer, AIDS, Alzheimer's disease, and inflammatory diseases (28,29). Two observations in this study demonstrate for the first time changes in glycosylation of GdA in GDM. First, the binding affinities of DGdA to ConA and WGA were lower than that of NGdA. Second, glycomics analyses of the *N*-glycans revealed substantive quantitative and qualitative differences in the glycan structures between NGdA and DGdA. The main quantitative difference is the smaller amount of α2-6 sialylated glycans in DGdA. An interesting qualitative difference is that most of the major sialylated glycans in NGdA appear as nonsialylated in DGdA.

Sialic acid levels on glycoproteins are regulated by sialyltransferases during their cellular biosynthesis and, in some instances, by sialidase(s) after secretion from cells. Decreases in sialyltransferase (30) and increases in sialidase activities (30,31) as well as changes in other glycosidase activities (32) have been reported in humans and animals suffering from diabetes. These findings are in accordance with the increased free sialic acid level in the serum of type 2 diabetes (33). Human endometrial tissues expresses both sialidase and sialyltransferase (27,34). In this study, placental sialidase activity is higher in GDM than in normal pregnancy, consistent with the reported abnormal carbohydrate metabolism in the placental-decidual unit of GDM pregnancy (35). Therefore, it is not surprising that the altered carbohydrate metabolism in GDM leads to the production of GdA with reduced sialic acid content.

Sialic acid is usually the terminal monosaccharide in human *N*-glycans, and it affects the conformation, binding, and biological activities of glycoproteins (29). The relative amount of some sialic acid–containing glycoproteins in amniotic fluid (36) and maternal plasma (37) is elevated during pregnancy and increases with advancing gestation. The results of this study emphasize the role of sialylation in pregnancy; a decrease in sialic acid content reduces the immunomodulatory activities of DGdA. Indeed, the abundance of sialic acid in different glycodelin isoforms correlates with their apoptosis-inducing activity on lymphocytes (11). Consistently, DGdA with less sialylated glycans has reduced apoptosis-inducing activity on lymphocytes, supporting the importance of sialic acid in mediating the immunomodulatory function of GdA.

In a normal pregnancy, selective deletion of T-cells occurs at the fetomaternal interface throughout gestation (38). Suppression of the response of maternal lymphocytes to fetal alloantigen is necessary for fetal survival (39). GdA modulates the T-cell population by inducing apoptosis of T-cells (11) and expression of Fas in Th-1 lymphocytes (40). The reduced ability of DGdA to induce T-cell apoptosis could be, at least in part, responsible for the observed increase of lymphocytes in the GDM patients (7,41). The involvement of carbohydrate metabolism in alteration of the T-cell population in GDM is reflected by the reduction of T-cells after insulin treatment of these women (7,41).

Changes in GdA glycosylation may also lead to inappropriate cytokine profiles in GDM. A shift in cytokine profile in women with GDM has been documented (6,7). Whether this is related to an increased risk of complications in GDM remains to be investigated. Significantly, T-cells treated with DGdA produce more IL-2 than those treated with NGdA. Excessive production of Th-1 cytokines including IL-2 would mediate rejection of the fetal semiallograft (42). On the other hand, DGdA has impaired

TABLE 5
Effect of NGdA, DGdA, and desialylated NGdA on IL-2 secretion of PBMCs and Jurkat cells and IL-6 secretion of pbNKs

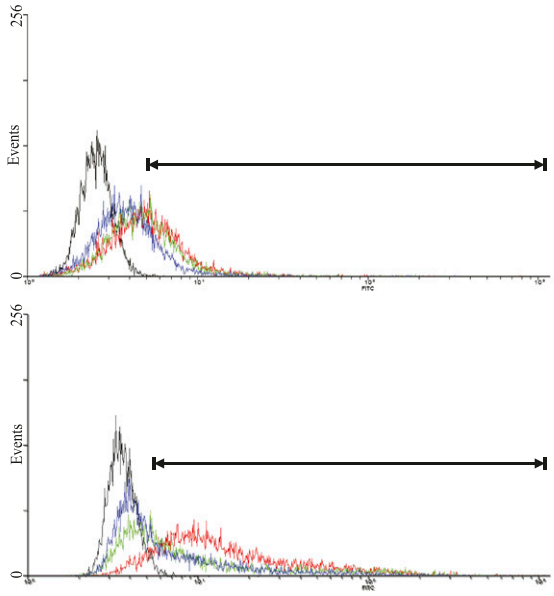
	IL-2 (pg/mL)		IL-6 (pg/mL)
	PBMCs	Jurkat cells	pbNK
Control			
GdA (μg/mL)			
0	1,045.2 ± 53.5	1,117.5 ± 138.3	30.1 ± 0.3
NGdA			
GdA (μg/mL)			
0.01	549.3 ± 42.5*	389.3 ± 130.6*	27.0 ± 0.8*
0.1	343.5 ± 113.9*	390.1 ± 59.6*	224.6 ± 130.7*
1	267.9 ± 87.8*	351.8 ± 99.8*	999.8 ± 294.7*
DGdA			
GdA (μg/mL)			
0.01	1,012.7 ± 35.5†	727.6 ± 78.3*†	52.3 ± 19.2
0.1	777.3 ± 108.6*†	567.2 ± 62.1*†	262.9 ± 104.1*
1	500.0 ± 160.3*†	435.7 ± 93.5*	287.1 ± 113.0*†
Desialylated NGdA			
GdA (μg/mL)			
0.01	1,037.6 ± 119.8†	1,123.8 ± 120.3†	76.6 ± 41.7
0.1	933.8 ± 88.9†	949.1 ± 74.9†	264.8 ± 142.4
1	875.0 ± 110.5*†	981.4 ± 78.3†	596.9 ± 139.0*†

Data are means ± SEM, $n = 5$. PBMCs (1×10^6) primed by PHA (5 μg/mL) and Jurkat cells were incubated with 0–1 μg/mL glycodelins for 16 h. pbNK cells (1×10^6) were incubated with 0–1 μg/mL glycodelins for 14 h. IL-2 and IL-6 secretions were quantified by ELISA. *$P < 0.05$ vs. the control without treatment. †$P < 0.05$ vs. NGdA at the same concentration.

stimulatory effect on IL-6 secretion by pbNK cells. IL-6 has a wide range of biological activities, including stimulation of trophoblast invasion (43) and hCG production (44). Inadequate IL-6 concentration in the placenta and endometrium has been associated with fetal growth restriction and recurrent miscarriage (45,46).

Both poorly sialylated DGdA and desialylated NGdA have impaired binding affinities to lymphocytes and pbNK cells. Sialic acid receptors, such as sialic acid–binding immunoglobulin-like lectin receptor, on leukocytes (29) have been proposed to mediate the action of glycodelin on B-cells (47). Consistently, the reported receptors of glycodelin isoforms on spermatozoa (10) and lymphocytes (48) are known to bind sialic acid–containing epitopes. The identity of the receptor(s) mediating the action of GdA on lymphocytes and pbNK cells remains unknown.

DGdA has proportionally more sialylated glycans with the Sda epitope (NeuAcα2–3[GalNAcβ1-4]Gal) and less high-mannose glycans compared with NGdA. Two observations suggest that these changes may not be related to the change in immunomodulatory activities of DGdA. First, two other glycodelin isoforms, namely glycodelin-F and



	GdA-bound cell (%)		
	PBMC	Jurkat	pbNK
N.GdA	35.7±5.3	72.1±4.2	72.6±4.4
D.GdA	27.6±6.0	52.7±4.8†	62.9±2.1†
Desialylated -N.GdA	15.4±4.6†	39.3±6.1†	44.9±2.0†

FIG. 2. The binding of NGdA, DGdA, and desialylated NGdA to PBMCs, Jurkat cells, and pbNK. PBMCs, Jurkat cells, and pbNK (1×10^6) were incubated with 1 μg/mL fluorescence-labeled mouse IgG (black), NGdA (red), DGdA (green), and desialylated NGdA (blue) for 2 h. GdA-bound cells were quantified by flow cytometry. Data are means ± SEM, $n = 5$. †$P < 0.05$ when compared with the NGdA at the same concentration. The results shown are representative of five replicate experiments.

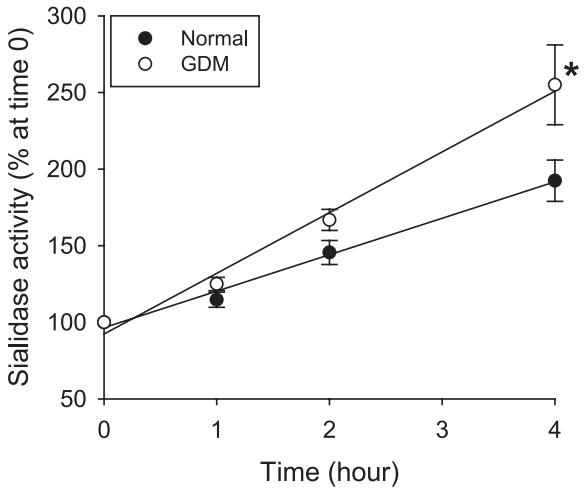


FIG. 3. Sialidase activity in the total cell lysates of normal and GDM placental tissues. Sialidase activity in the total cell lysates of normal (n = 10) and GDM placental tissue (n = 10) was determined by incubation with 4MU-NANA at pH 4.5 for 1–4 h at 37°C. The results represent the means ± SEM and are expressed as percentage of activity at time 0. *$P < 0.05$ when compared with the normal placental tissue at the same time point.

glycodelin-C, also carry the Sda epitopes, but only the former has immunomodulatory activity (11). Second, another glycodelin isoform, glycodelin-S, contains more high-mannose glycans than GdA (10), but glycodelin-S is not immunosuppressive (11). Additional investigation is required to understand the biological implication of these glycosylation changes.

In conclusion, this study provides the first direct evidence that changes in the glycosylation of decidual glycoprotein GdA is associated with defective binding and immunomodulatory activities of this molecule. These discoveries may give a new lead to the study of protein glycosylation in the pathophysiology of GDM. It is possible that the changes in GdA glycosylation in GDM are related to increased placental sialidase activity and, therefore, are not applicable to all types of diabetes. It also remains to be seen whether the changes described herein have any connections with fetal complications or have clinical consequences of altered immune cell reactivity. In type 2 diabetes, an increase in serum sialic acid levels is an indication of the loss of sialylation from circulatory and membrane glycoproteins (33). Approaches aimed at fixing the glycosylation changes may help to alleviate some of the complications associated with GDM. In this connection, the glycosidase inhibitor miglitol used to treat type 2 diabetes (49) has been shown to modify the *N*-linked glycosylation of secretory glycoproteins (50). The application of the MS-based glycomics strategies described herein will also open a new avenue for understanding the association of structural and protein-specific glycosylation in diabetes and its associated pathological conditions.

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