Congenital disorder of fucosylation type 2c (LADII) presenting with short stature and developmental delay with minimal adhesion defect

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Leukocyte adhesion deficiency type II is a hereditary disorder of neutrophil migration caused by mutations in the guanosine diphosphate-fucose transporter gene (SLC35C1). In these patients, inability to generate key fucosylated molecules including sialyl Lewis X leads to leukocytosis and recurrent infections, in addition to short stature and developmental delay. We report two brothers with short stature and developmental delay who are compound heterozygotes for novel mutations in SLC35C1 resulting in partial in vivo defects in fucosylation. Specifically, plasma glycoproteins including immunoglobulin G demonstrated marked changes in glycoform distribution. While neutrophil rolling on endothelial selectins was partially impeded, residual adhesion proved sufficient to avoid leukocytosis or recurrent infection. These findings demonstrate a surprising degree of immune redundancy in the face of substantial alterations in adhesion molecule expression, and show that short stature and developmental delay may be the sole presenting signs in this disorder.

INTRODUCTION

Innate immunity critically depends on the capacity of circulating granulocytes to migrate efficiently to sites of infection. Recruitment begins with tethering and rolling interactions between granulocytes and target tissue endothelium. These shear–resistant interactions are principally mediated by the selectins, a family of calcium-dependent lectins. The prototypical binding determinant for selectins is the fucosylated glycan known as sialyl-Lewis X (sLex/CD15s).

A hereditary deficiency of fucosylation has been described in humans. In this condition, known as leukocyte adhesion deficiency type II (LADII) (MIM 266265), affected individuals bear mutations in the Golgi transporter that shuttles the nucleotide sugar precursor, guanosine diphosphate (GDP) fucose, from the cytosol where it is made, to the Golgi where it is used to create fucosylated glycoconjugates (1–6). Accordingly, LADII patients are unable to generate fucosylated glycans including CD15s. This results in the inability of granulocytes to bind selectins which manifests with marked leukocytosis, an inability to
generate pus, and recurrent bacterial infections. These clinical manifestations of impaired immunity are the cardinal signs of LADII. Besides deficiencies in host defense, LADII patients lack the fucosylated Lewis blood group and ABO antigens resulting in the rare Bombay blood type (7). Importantly, though deficits in host defense prompted the initial discovery of this condition, patients with LADII also suffer from growth retardation, developmental delay and dysmorphic features (8), but it is not clear how lack of fucosylation yields these phenotypes. Due to these diverse manifestations, this disorder has also been termed congenital disorder of glycosylation type 2c (CDG2c). From prior clinical observations, it is believed that all physical manifestations of LADII require a severe defect of glyconjugate fucosylation. We report here on brothers who have developmental delay and significant growth retardation and on the genetic, structural and functional studies undertaken to elucidate the biochemical basis of their condition. The results of our studies of these patients, at the nexus of genomics and glycomics, provide new perspectives on the molecular basis of defects of leukocyte migration and host defense, and force reconsideration of the assessment of children who present with growth and developmental compromise.

Case presentations

Our patients are two brothers, now 21 (Proband 1) and 19 (Proband 2) years of age, born to non-consanguineous parents of British heritage with unremarkable family history. The pregnancies and births were uncomplicated. Proband 1 had a birth weight of 2.5 kg (<2nd %) and length of 49.5 cm (60th %), and Proband 2 had a birth weight of 3.0 kg (25th %) and length of 51 cm (70th %). Their 13-year-old sister has had normal growth and development.

The brothers have global developmental delay; however, expressive language and cognition are the more significant challenges. Proband 1 currently has an ~150 word vocabulary, speaking in three word phrases with echolalia; he has anxiety and is on the autism/pervasive developmental disorder spectrum. Proband 2 has less global delays, milder autistic features and obsessive–compulsive disorder.

Their past medical history is significant for multiple episodes of otitis media as infants which responded to oral antimicrobial therapy. Neither had placement of tympanostomy drainage tubes, but Proband 2 had an adenoidecotomy secondary to episodic sinus infections. Proband 1 had a hospitalization at age 12 weeks for bronchiolitis and at age 10 months for a febrile seizure, but otherwise neither brother had any serious infections. Comprehensive endocrine and metabolic evaluation revealed normal serum IGF-I and IGFBP-3 levels, plasma amino acids, urine amino acids and organic acids, urine sialic acid, mucopolysaccharides and urine oligosaccharides. Chromosomal microarrays showed no copy number variants or areas with loss of heterozygosity. Notably, white blood cell counts and absolute neutrophil counts were within the normal range on two occasions (Supplementary Material, Table S1) and both brothers had the O+ blood type. Phenotypically, both brothers have short stature with heights of −2.8 and −3.3 standard deviations, coarse facial features with a bulbous nose, widow’s peak, small hands and feet with brachydactyly and persistent fetal finger pads (Fig. 1).

RESULTS

Targeted sequencing of the exons of 1077 candidate genes was performed on Proband 1 as part of a short stature research study (9). Proband 1 was found to carry two novel non-synonymous variants in SLC35C1, the gene which encodes the Golgi GDP-fucose transporter 1 (UniProt Q96A29). Sanger sequencing was performed in both subjects, their parents and unaffected sister. The subjects’ mother carries the p.Glu31* (c.91G>T) nonsense variant while the father carries the p.Phe168del (c.501_503delCTT) variant. Both subjects were confirmed to be compound heterozygotes for the two variants and the unaffected sister does not carry either variant. The nonsense variant abrogates essentially the entire protein including all the transmembrane domains while the Phe168del variant leads to loss of a single phenylalanine which is the second amino acid in the 5th transmembrane domain (10).

As the subjects were missing some of the key features of LADII, namely leukocytosis, recurrent infections and the Bombay blood type, we sought to ascertain whether their
variants in SLC35C1 were associated with abnormalities in glycosylation. To this end, we evaluated an abundant circulating glycoprotein, immunoglobulin G (IgG). Approximately 95% of the glycans on IgG feature a fucose attached to the first N-acetylglucosamine linked to asparagine in N-glycans (11,12). We hypothesized that defective GDP-fucose transportation from the cytosol into the Golgi would alter this pattern. While glycans from control subjects showed the expected marked predominance of fucosylated forms, both probands exhibited approximately equal proportions of fucosylated and non-fucosylated IgG heavy chain glycans, consistent with a marked but partial fucosylation defect (Fig. 2). To assess whether this abnormality reflected defective fucosylation alone and not a broader abnormality in IgG glycosylation, we treated patient and control glycans with α-L-fucosidase. After enzymatic defucosylation, patient and control glycans were indistinguishable, confirming that the defect was selective to fucosylation (Fig. 2; Supplementary Material, Fig. S1). To assess whether this fucosylation defect was selective to the affected family members, we tested IgG heavy chain glycans from the unaffected father, mother and sister. These resembled controls and were markedly distinct from the affected cases, showing that presence of both mutant alleles was required to elicit the phenotype (Fig. 2).

We then proceeded to perform a global plasma N-glycan analysis using mass spectrometry (Supplementary Material, Fig. S2). This confirmed that, in contrast to their unaffected family members, the two subjects had global increases in non-fucosylated glycans, but each was able to make fucosylated glycans. Peaks consistent with the H2 antigen and sialyl-Lewis X were present.

**Figure 2.** Characterization of N-glycans from purified IgG heavy chains by normal-phase HPLC. (A) N-Glycans were extracted from purified heavy chains of Probands 1 and 2 (green and red) and from male age matched pediatric controls undergoing evaluation for short stature (black, blue and gray). HPLC tracings normalized to the G1F/ G1FB peak reveal that patients 1 and 2 show a striking abundance of afucosylated species (G0, G1, G2), whereas these species represent only a minor fraction of IgG glycans among controls. The letters reflect the specific glycan species shown, including the number of galactoses (G, 0–2), core-fucosylation if present (F) and the presence of a bisecting N-acetylglucosamine if present (B). For example, G1FB defines a monogalactosylated structure with both a core fucose and a bisecting N-acetylglucosamine. (B) HPLC tracings from the same patients and controls following defucosylation, demonstrating normalization of differences; see Figure S1 for before and after tracings from representative patients. (C) Quantitation of non-fucosylated and fucosylated species among agalactosyl (G0 + G0F) IgG heavy chain glycans. *P < 0.05, **P < 0.001 by Student’s t-test. (D) Glycan profiles from patients compared with unaffected family members (mother M, turquoise; father F, purple; sister S, pink). A TSKgel amide-80 column (4.6 mm ID × 15 cm, 3 μm) was used for the separation in (A)–(C), while a TSKgel amide-80 column (4.6 mm ID × 25 cm, 5 μm) was used for separation in (D), accounting for differences in trace profiles.
To further explore the hematological phenotype, we performed flow cytometry and western blot analysis, as well as parallel plate flow chamber assays of peripheral blood granulocytes obtained from affected and unaffected family members. Granulocytes were analyzed for expression of CD15s, and were also analyzed for expression of P-selectin glycoprotein ligand-1 (PSGL-1), the principal CD15s-bearing glycoprotein on the granulocyte surface. Whereas >95% of granulocytes from unaffected family members expressed CD15s, 88 and 50% of granulocytes from the two probands displayed CD15s with a mean expression level per cell profoundly lower (>20-fold) than in unaffected family members. However, there was no appreciable difference in expression of PSGL-1 among family members. Western blot confirmed the relative absence of CD15s display on PSGL-1 (Fig. 3) on granulocytes of probands.

Granulocytes from all family members were perfused over TNFα-stimulated human umbilical vein endothelial cell (HUVEC) monolayers to assess E-selectin-mediated tethering and rolling under increasing shear stress. In each case, specificity for binding to E-selectin was assessed by use of anti-E-selectin blocking antibody. While granulocytes of probands were capable of tethering and rolling adhesive interactions, the total numbers of granulocytes from unaffected family members which tethed and rolled at all shear stresses (0.5–10 dyne/cm²) were considerably higher. Notably, at shear stress of ≥2 dyne/cm², granulocytes from unaffected family members maintained rolling interactions with the HUVEC monolayers while those from affected subjects did not. Furthermore, analysis of rolling velocities revealed that granulocytes from the probands rolled significantly faster, indicating weaker attachment to E-selectin (Fig. 3; Supplementary Material, Movie).

DISCUSSION

In 1992, Etzioni et al. first reported two cases of LADII (2,8). These two patients presented with recurrent severe bacterial infections in infancy. In total, there have been seven reported cases of
LADI (2,7,8,10,13,14), and while this disorder is quite rare, it has provided fundamental insights into the glyobiology of leukocyte migration and fucose metabolism. To date, all subjects with LADI share the key clinical characteristics of significant neutrophilia, the Bombay blood type, as well as short stature and marked mental retardation (15). In each case, the presenting clinical symptom prompting evaluation was recurrent bacterial infection, thus drawing attention to deficits in innate immunity. Here we report two brothers in whom the presenting signs prompting evaluation were short stature and developmental delay. Though each manifested episodes of otitis media, neither the frequency nor intensity of bacterial infections raised a suspicion of neutrophil dysfunction. Genomic studies revealed that they are compound heterozygotes for two non-synonymous variants in SLC35C1, the locus encoding the GDP-fucose transporter. Consistent with classic LADI, they have short stature and mental retardation but they lack the distinctive Bombay blood type and neutrophilia. N Glycan analysis demonstrates a global decrease but not absolute lack of fucosylation with the presence of the H (Bombay) antigen and CD15s. Likewise, granulocyte flow cytometry studies show a marked decrease in surface CD15s, resulting in diminished but not absent granulocyte rolling on vascular E-selectin. Notably, the canonical leukocyte P- and E-selectin ligand, PSGL-1, showed normal expression by western blot but no expression of the CD15s determinant.

The Bombay blood type, one of the key diagnostic features for LADI, is due to the absence of the fucosylated H antigen. All patients reported to date with classic LADI presented with the Bombay blood type. We hypothesize that our patients’ lack of the Bombay blood type is due to a less severe partial defect in fucosyl transport. This presumably allows them to produce some amount of the H antigen, as seen in the global N-glycan analysis, which leads to lack of anti-ABO blood group antibodies which characterize the Bombay blood type.

It is striking that despite low CD15s expression and clear defects in granulocyte rolling, the subjects did not present with clinical features of severe recurrent infections or neutrophilia. There are two possible explanations for this finding. The simplest explanation is that the minimal expression of CD15s present in the brothers allows for sufficient leukocyte rolling to achieve immune surveillance. This possibility is most likely, as the results of parallel plate flow assays show that proband granulocytes are capable of tethering and rolling interactions at the lower end of physiologic shear stress (1–4 dynes/cm²) found within post-capillary venules, the vascular portal of extravasation (16). The other possibility is that adaptive immunity was sufficient to protect these patients from infections and plays a more prominent role in preventing infections as these patients age. Notably, patients with classic LADI do seem to outgrow their recurrent infections presumably due to compensatory mechanisms, although the granulocytosis never completely resolves (15). Nonetheless, our data suggest that considerable immune competence can be achieved with significantly depressed levels of E-selectin ligand activity challenging existing paradigms in leukocyte trafficking.

The data presented here draw attention to the spectrum of biologic changes engendered by fucosylation defects. In contrast to canonical LADI, the probands lacked the hematological phenotype, but did present with growth and mental retardation. The mechanism of short stature and development delay is not well understood in LADI. Fucosylated glycans are known to play a role in brain development and presumably disruption of these glycans could lead to developmental delay (17,18). Notch signaling is critical for brain development and N-fucosylation has been shown to be severely disrupted in fibroblasts from a patient with LADI (19). Additionally, both the insulin-like growth factor 1 receptor (20,21) and the epidermal growth factor receptor (22) are known to be fucosylated. Defects of fucosylation in both of these pathways which are related to growth have been demonstrated to lead to post-receptor signaling defects in mouse models (22,23).

Previously, identification of immune deficits was key for consideration of the diagnosis of LADI. Our studies unveil a previously unappreciated spectrum of presentation of fucosylation defects, indicating that the prior emphasis on immune deficits may underestimate the prevalence of this condition within the general population. Short stature and developmental delay may be the sole presenting signs of this disorder. The identification of fucosylation defects in our patients has critical ramifications in so far as two patients with classic LADI responded to fucose supplementation (7,14) including one young child with improvement in cognitive development (7). Had this diagnosis been made in infancy, it is possible that our subjects’ growth and mental retardation could have been improved with fucose supplementation. Quantitation of granulocyte CD15s expression via flow cytometry is a readily translatable low-cost diagnostic approach for this disorder. While likely to be a rare cause of short stature, our case demonstrates that children who present with growth and developmental compromise should raise consideration of screening for fucosylation defects even in the absence of signs and symptoms of classic LADI.

Materials and Methods

This study was approved by the institutional review board at Boston Children’s Hospital. Written informed consent was obtained from subjects or their legal guardians.

Sequencing methods

DNA samples from multiple subjects were pooled for DNA sequencing using previously described methods available at the Broad Institute (24). The targeted exons of the 1077 candidate genes were enriched using a custom Agilent SureSelect hybrid selection system. The candidate genes selected comprise genes from identified genome-wide association loci associated with height as well as genes known to cause skeletal dysplasias or syndromic conditions which include short stature as previously described (9). After hybrid selection, barcoded libraries were prepared for each pool. Sequencing was performed on the Illumina HiSeq platform. Syzygy software (24) was used to call variants. Variants were annotated for functional effect using Snpeff 2.0.5 (http://snpeff.sourceforge.net/). Variant allele frequency data were obtained from two publicly available data sets: (i) integrated variant call set of 1000 Genomes Phase 1 samples (25) (February 2012 release) and (ii) National Heart Lung and Blood Institute Exome Variant Server (26). Novel variants were not observed in these two datasets or in dbSNP. SLC35C1 variants were annotated using reference sequences
NM_018389.4 and NP_060859.4. Primers for Sanger sequencing are available on request.

Characterization of IgG heavy chain N-glycans using normal-phase HPLC separation

N-Glycan analysis was performed according to the procedure detailed by Royle et al., with slight modifications (27,28). IgG heavy chains were isolated from the plasma of patients and relevant controls. IgG was purified using a protein G column and then reduced, denatured and separated on a 12% discontinuous SDS–PAGE gel. Heavy bands were excised and N-glycans released by overnight incubation in PNGase F at 37°C. The released N-glycans were collected and labeled with 2-aminobenzylamine (2AB). Excess label was removed by absorbing the mixture of free and glycan-conjugated 2AB on 3MM Whatman chromatography paper. After washing away the unreacted 2AB with acetonitrile, labeled glycans were released with water. Dried and resuspended samples were separated on a Waters 1525 binary HPLC pump equipped with a Waters 2475 fluorescence detector with inline TSKgel amide-80 column (4.6 mm ID × 15 cm, 3 μm beads or 4.6 mm ID × 25 cm, 5 μm beads) with a linear gradient of 35–47% 50 mM formic acid (pH 4.4) while decreasing acetonitrile after 48 min.

Fucosidase treatment

The 2AB-labeled glycans in water were supplemented with phosphate buffer (pH 5.5) to a final concentration of 50 mM. The mixture (20 μl) was treated with α-L-fucosidase from bovine kidney (specific activity, ≥2 units per mg protein, Sigma-Aldrich) overnight at 37°C. Defucosylated glycans were characterized as described above.

Plasma global N-glycan analysis using mass spectrometry

For plasma analysis, 200 μl was added to a glass test tube. Glycosphingolipids were extracted with 2:1 chloroform/methanol. The protein pellet was digested with trypsin/chymotrypsin as described (29). N-Linked glycans were released using N-glycanase (Prozyme) and separated via C18 and porous graphitized carbon solid phase extraction (30). The purified N-glycans were reduced and permethylated as described (31). Mass spectrometry analysis was performed on an LTQ (Thermo-Fisher, San Jose, CA, USA) equipped with a Nanomate nano-electrospray source (Advin, Ithaca, NY, USA). Samples were dissolved in 1:1 methanol/water. All ions are sodium adducts. Peaks were selected manually for MS^n analysis.

Peripheral blood granulocyte isolation

Whole peripheral blood from all the family members was obtained. The components of the peripheral blood were separated using gradient centrifugation over Histopaque-1077 (Sigma). After centrifugation at 400 g for 30 min, the granulocytes were collected from the buffy coat layer above the red blood cell pellet. The granulocytes were treated briefly (<30 s) with Red Blood Cell Lysing Buffer (Sigma) with further washes in PBS w/o Ca^2+/Mg^2+.

Western blot

Granulocytes were lysed with 2% NP-40 in 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 20 μg/ml PMSF, 0.02% sodium azide and protease inhibitor cocktail tablet (Roche Molecular Biochemicals). The cell lysates were normalized based on cell numbers and were diluted with Laemmli reducing sample buffer and resolved using 4–20% SDS–PAGE gels (Bio-Rad). Membrane proteins were then transferred to Sequi-blot polyvinylidene difluoride membrane (Bio-Rad), blocked and stained with either HECA452 (Biolegend) or KPL-1 (BD Biosciences), then washed with Tris-buffered saline/0.1% Tween 20. Blots were then incubated with appropriate horse radish peroxidase-conjugated secondary antibodies (anti-RatIgM for HECA452 or anti-MsIgG for KPL-1), washed and visualized with chemiluminescence using Lumi-Light Western Blotting Substrate (Roche Diagnostics).

Flow cytometry

Aliquots of cells were washed with PBS/2% FBS and incubated with primary mAbs or with isotype control mAbs. The cells were washed in PBS/2% FBS and, for indirect immunofluorescence, incubated with appropriate secondary fluorochrome-conjugated antibody. After washing the cells, fluorescence intensity was determined using a Cytomics FC 500 MPL flow cytometer (Beckman Coulter).

Parallel plate flow chamber assay

A parallel plate flow chamber (GlycoTech) was mounted on top of a confluent monolayer of TNF-α-activated HUVEC (obtained from the Vascular Biology Core, Brigham & Women’s Hospital). Purified granulocytes, resuspended in binding buffer (HBSS, 10 mM HEPES, 2 mM CaCl_2) at a concentration of 1 × 10^6cells/ml, were then perfused over the HUVEC monolayer under defined shear stress conditions by a syringe pump (Harvard Apparatus). After introduction of the granulocytes into the chamber at 1 ml/min, the flow was momentarily suspended, allowing for cells to contact the HUVEC monolayer. Next, the flow rate was then increased incrementally to exert shear stress at levels from 0.25 to 10 dynes/cm^2. Cell–cell interactions were monitored and recorded in real time with an inverted phase contrast microscope, connected to a video camera, an SMPTE time code generator, a monitor and a DVD recorder. Recorded videos were analyzed offline. In each case, specificity for binding to E-selectin was assessed by use of anti-E-selectin blocking monoclonal antibody. The number of leukocytes rolling were quantified under ×100 magnification at shear stresses of 0.5, 1, 2, 5 and 10 dynes/cm^2 with four independent fields of view (FOV) of 15 s intervals for each shear stress. The rolling velocities were determined by measuring the rolling distance and time traveled by a granulocyte.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.
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Conflict of Interest statement. None declared.

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