Conditional control of selectin ligand expression and global fucosylation events in mice with a targeted mutation at the FX locus

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Glycoprotein fucosylation enables fringe-dependent modulation of signal transduction by Notch transmembrane receptors, contributes to selectin-dependent leukocyte trafficking, and is faulty in leukocyte adhesion deficiency (LAD) type II, also known as congenital disorder of glycosylation (CDG)-IIc, a rare human disorder characterized by psychomotor defects, developmental abnormalities, and leukocyte adhesion defects. We report here that mice with an induced null mutation in the FX locus, which encodes an enzyme in the de novo pathway for GDP–fucose synthesis, exhibit a virtually complete deficiency of cellular fucosylation, and variable frequency of intrauterine demise determined by parental FX genotype. Live-born FX(−/−) mice exhibit postnatal failure to thrive that is suppressed with a fucose-supplemented diet. FX(−/−) adults suffer from an extreme neutrophilia, myeloproliferation, and absence of leukocyte selectin ligand expression reminiscent of LAD-II/CDG-IIc. Contingent restoration of leukocyte and endothelial selectin ligand expression, general cellular fucosylation, and normal postnatal physiology is achieved by modulating dietary fucose to supply a salvage pathway for GDP–fucose synthesis. Conditional control of fucosylation in FX(−/−) mice identifies cellular fucosylation events as essential concomitants to fertility, early growth and development, and leukocyte adhesion.

Introduction

Mammalian cell surface glycans are characterized by enormous structural diversity and by dynamic tissue-specific and developmentally regulated expression patterns. Rare carbohydrate-deficient glycoprotein syndromes accounted for by mutations in loci that control glycosylation, demonstrate that deficient or aberrant glycosylation pathways are associated with serious and widespread disruptions in organ physiology (Freeze, 2001). Similarly, mice with induced null mutations in genes that determine glycosylation pathways inform us that complex-type N-glycans are required for normal mammalian development (Metzler et al., 1994), for example, and that several distinct, precisely controlled, tissue- or cell type–specific terminal modifications of O-glycans are required for immune system function (Lowe, 2001). Nonetheless, functional correlates remain to be assigned to the majority of glycan structural diversity in mammals.

Fucose is a monosaccharide found widely in the plant and animal kingdoms (Staudacher et al., 1999). In humans and other mammals, fucose is a component of many surface-localized and secreted molecules, where it decorates the terminal portions of N-, O-, and lipid-linked glycans, modifies the core of some N-linked glycans, and is linked covalently to some serine and threonine residues (Fig. 1). Fucosylation of mammalian glycans is catalyzed by distinct fucosyltransferases, numbering roughly a dozen or fewer in each mammalian species, with catalytic activities characterized by precise specificity for glycan precursors and a requirement for the sugar nucleotide substrate GDP–fucose (Fig. 1; Oriol et al., 1999). Terminal α(1,2)-fucosylated glycans in humans contribute to the ABO, Secretor, and Lewis blood group antigens (Lowe, 2000). In the gastrointestinal tract, fucosylation-dependent microbe–epithelial cell interactions are assigned a role in the attachment and pathogenesis of Helicobacter pylori infection (Hooper and Gordon, 2001), and in a commensal relationship between the anaerobic bacterium Bacteroides thetaiotaomicron and its rodent host (Hooper and Gordon, 2001). Fucosylation of the glycans that decorate the leukocyte and endothelial cell counter receptors for the selectin family of cell adhesion...
molecules are prominent determinants of the activities of these counter receptors, and thereby control selectin-dependent leukocyte adhesion and trafficking events in the mammal (Homeister et al., 2001). Fucose also represents an apparently essential component of glycans that decorate the Notch family of transmembrane receptors, and whose structures are determined in part by the Fringe family of glycosyltransferases. These fucose-dependent glycans can modulate signal transduction events initiated by interactions between Notch and its ligands (Bruckner et al., 2000; Moloney et al., 2000b).

Aberrant fucosylation is associated with pathophysiology in leukocyte adhesion deficiency (LAD)* type II, also known as congenital disorder of glycosylation (CDG) type IIc (Freeze, 2001), a rare human disorder characterized by psychomotor defects, developmental abnormalities, and leukocyte adhesion defects (Erzioni et al., 1992; Frydman et al., 1992; for review see Becker and Lowe, 1999). Missense mutations in a Golgi-localized GDP–fucose transporter account for defective fucosylation in these individuals (Lubke et al., 2001; Luhn et al. 2001). Based on our understanding of the contribution by fucosylation to the activities of leukocyte selectin counter receptors (Homeister et al., 2001), the leukocyte adhesion defect in LAD-II/CDG-IIc can be clearly assigned to defective fucosylation. Nonetheless, the pathophysiological basis for the many developmental, psychomotor, and morphological abnormalities in these patients (Freeze, 2001) remains mysterious.

Intentional manipulation of glycan fucosylation by modulating fucosyltransferase expression in cellular contexts (Lowe, 2000) or chronically and irreversibly in the context of an intact mammal (Domino et al., 2001; Homeister et al., 2001) have proven informative in the discovery of functions for fucosylated glycans. Similarly, an ability to control cellular and organ fucosylation intentionally, but acutely and reversibly, may advance efforts to unveil functions for fucosylated glycans in novel contexts, to assign function in contexts where fucosylated glycans are inferred to contribute to development (Feizi, 1985; Fenderson et al., 1986) and fertility (Dell et al., 1999), and to further define contributions made by fucosylated glycans in the previously explored contexts discussed above. Towards these ends, we report here the construction and characterization of mice with an induced mutation in the locus encoding GDP-4-keto-6-deoxymannose 3,5-epimerase-4-reductase (also known as FX) (Szikora et al., 1990; Cam-

Abbreviations used in this paper: CDG, congenital disorder of glycosylation; CFU, colony-forming unit; dpc, days postcoitum; ES, embryonic stem; GM, granulocyte–macrophage; GMD, GDP-mannose 4,6-dehydratase; HEV, high endothelial venule; LAD, leukocyte adhesion deficiency; M/E, myeloid–erythroid; PSA, *Pisum sativum* agglutinin; WT, wild-type.
ardella et al., 1995; Tonetti et al., 1996), an enzyme thought to be essential to the constitutive and primary pathway for the synthesis of the fucosyltransferase substrate GDP–fucose (Becker and Lowe, 1999). In the adult mouse, homozygosity for the FX-null allele confers a virtually complete dependence on a fucose-dependent salvage pathway for GDP–fucose synthesis, enables intentional, conditional, and reversible control of glycan fucosylation in the animal’s cells and organs, and reveals a requirement for fucosylated glycans in fertility, growth and development, leukocyte adhesion, homeostasis in the gastrointestinal tract and hematopoietic physiology.

Results

The FX(−/−) genotype is associated with a partially penetrant embryonic lethal phenotype

Embryonic stem (ES) cell transfectants containing homologous integration of the targeting vector were identified by Southern blot analyses (Fig. 2). Male chimeras derived from correctly targeted ES clones generated progeny heterozygous for the mutant FX allele, from heterozygous male and female progeny of chimeric mice derived from ES clone FXD3D (lanes +/− Male and +/− Female), and from littermates derived from this breeding triangle (Progeny). Blots were hybridized with probe A (top), stripped, and rehybridized with probe C (bottom). Fragments corresponding to the WT and 3,5-epimerase/4-reductase alleles migrate at 8.9 and 5.7 Kb, respectively. Reference fragments are designated in kb.
eight FX(-/-) mice (three males, three females) were identified in 360 progeny genotyped at weaning (Table I). A slight deficit of heterozygotes was observed, relative to the expected Mendelian distribution, indicating a partial loss of heterozygotes and a selective and profound loss of homozygous-null embryos. Among embryos collected from females impregnated in timed matings, a normal Mendelian distribution was observed at 3.5 d postcoitum (dpc). Thereafter, a relative loss of FX(-/-) progeny was observed at 12.5 dpc, and a further loss at 16.5 dpc, such that immediately prior to birth, the number of FX(-/-) embryos was 28% of the predicted number (Table II). A further and substantial apparent loss of FX(-/-) progeny was also evident during the weaning period (Table II).

Considered together with the predicted biochemical defect in the mice (Fig. 1), these observations suggested that absence of fucosylated glycans is associated with and perhaps accounts for the embryonic lethality. In an effort to circumvent the embryonic lethal phenotype by reconstitution of fucosylation via the salvage pathway for GDP-fucose synthesis, mating, gestation, and postpartum activities were completed in cages with water supplemented to 100 mM fucose. The frequency of null progeny in these matings at weaning (Table III) was essentially identical to that observed without fucose supplementation, or when matings were done with females gestated with diets supplemented with fucose only in chow, or in both chow and water (unpublished data). These observations indirectly indicate that dietary fucose is not able to reconstitute fucosylation in FX(-/-) embryos to a degree that permits their survival to term. Fucose

Table I. Genotype of progeny of FX(+/-) x FX(+/-) intercross

| Genotype | Male | Female | Total | % Observed | % Expected |
|----------|------|--------|-------|------------|------------|
| +/-      | 58   | 69     | 127   | 35.3       | 25         |
| +/-     | 116  | 111    | 227   | 63.0       | 50         |
| +/-     | 3    | 3      | 6     | 1.7        | 25         |

No fucose was added to chow or water. Genotype of progeny mice were determined at weaning (postnatal day 21). Chi-square analysis was used to compare the observed values with values predicted by Mendelian inheritance patterns. P < 10^(-3).

Table II. Genotypes of embryos derived from FX(+/-) x FX(+/-) intercross

| Days postcoitus | Genotype | 3.5 | 12.5 | 14.5 | 16.5 | 18.5 | Weaning |
|-----------------|----------|-----|------|------|------|------|---------|
| +/-             | 19%      | 30% | 37%  | 31%  | 29%  | 35.3% |
| +/-             | 54%      | 55% | 50%  | 64%  | 64%  | 63%   |
| +/-             | 27%      | 15% | 13%  | 5%   | 7%   | 1.7%  |
| n               | 52       | 130 | 109  | 125  | 113  | 360   |
| P value         | 0.630    | 0.036 | 0.002 | <10^(-3) | <10^(-3) | <10^(-3) |

No fucose was added to chow or water.

aPostnatal day 21.
bChi-square analysis was used to compare the observed values with the values predicted by Mendelian inheritance patterns.

Figure 3. Expression of fucosylated glycans in the spleen in FX(-/-) embryos. WT or FX (-/-) embryos were obtained at 18.5 d dpc from a mating between an FX (+/-) male and an FX(+/-) female reared without fucose supplementation. Fuc-TIV(-/-)/Fuc-TVII(-/-) 18.5 dpc embryos were derived from Fuc-TIV(-/-)/Fuc-TVII(-/-) intercrosses (Homeister et al., 2001). Frozen sections of the embryonic spleens were stained (Materials and methods) with anti–Gr-1 antibody (top). An adjacent section was stained with an E-selectin–IgM chimera in buffer containing 2 mM CaCl2 (middle) or in buffer containing 5 mM EDTA (bottom). EDTA-resistant staining observed with the E-selectin–IgM chimera is due to the secondary anti–human IgM used to detect the chimera (unpublished data). (10× final magnification).
supplementation during the postterm/prenweaning interval also does not significantly enhance survival to weaning, as indicated by a comparison of the data on the survival rate to weaning in Tables I and III, and by experiments in which the rate of survival of FX(−/−) mice between days 7 and 21 postterm was observed to be 100% in cages with (n = 14) or without (n = 10) fucose supplementation.

Fucosylated glycans are present in FX(−/−) embryos, but are not synthesized by FX(−/−) cells

In an effort to understand the apparent inability to rescue FX(−/−) embryos with maternal fucose supplementation, we sought to determine if fucosylated glycans are present in FX(−/−) embryos collected from FX(+/−) mothers reared and gestated in the absence of fucose. Glycans that contribute to fucosylation-dependent E-selectin ligand activities on mouse neutrophils (Maly et al., 1996; Homeister et al., 2001) were used to assess fucosylation at 18.5 dpc in the spleens of wild-type (WT) embryos and FX(−/−) embryos, and in the spleen of a strain of doubly fucosyltransferase-deficient mice that maintains a WT FX locus (Fig. 3; Homeister et al., 2001). An E-selectin–IgM immunohistochemical probe identifies such ligands in the neutrophil-rich (Gr-1–positive) spleens of WT embryos, whereas the chimera does not bind to the spleen in the fucosyltransferase-deficient embryo. In an FX(−/−) embryo, splenocytes retain E-selectin–IgM chimera binding. Identical results have been observed in both other independently studied FX(−/−) embryos (unpublished data).

Therefore, synthesis of fucosylated glycans can proceed in at least some FX(−/−) embryos in utero, even if the heterozygous mother is gestated on a standard diet. This observation is consistent with the possibility that the partially penetrant embryonic lethality associated with this genotype may be accounted for by fucosylation-independent mechanisms, or that subnormal fucosylation not detected by immunohistochemical methods contributes to decreased intrauterine viability (see Discussion). These observations do not exclude the possibility that fucosylation competency varies, genetically or otherwise, among FX(−/−) embryos, and that fucosylation competency may correspond to and account for survival in utero because we may only have sampled fucosylation competent embryos destined for survival, and not fucosylation incompetent embryos, either by chance, or because these perish prior to the 18.5 dpc sampling time.

Fibroblasts from FX(+/−) embryonic fibroblasts (Fig. 4) retained an intact cell surface fucosylated glycan expression (Kornfeld et al., 1981), binds to FX(+/−) (unpublished data) embryonic fibroblasts, but does not bind to FX(−/−) embryonic fibroblasts grown in the absence of exogenous fucose (Fig. 4). Nonetheless, FX(−/−) fibroblasts retain an intact salvage pathway for GDP–fucose synthesis and are competent to construct cell surface fucosylated glycans, as PSA binding is restored when they are grown in media supplemented with fucose (Fig. 4). These observations confirm that nullizygosity for the FX locus disables the constitutive pathway for synthesis of GDP–fucose from GDP–mannose, and diminish but do not exclude the possibility that an alternative fucose-independent pathway for GDP–fucose synthesis accounts for the fucosylated cell surface glycans detected in some FX(−/−) embryos. These results also imply that the GDP–fucose salvage pathway in such embryos may be nourished in utero by maternally or fraternally derived fucose or fucosylated glycans.

| Genotype      | Male | Female | Total | % Observed | % Expected |
|---------------|------|--------|-------|------------|------------|
| +/+           | 33   | 26     | 59    | 55.1       | 25         |
| +/−           | 18   | 28     | 46    | 43         | 50         |
| −/−           | 2    | 0      | 2     | 1.9        | 25         |

Cage water was supplemented with fucose to a final concentration of 100 mM. Genotypes of progeny mice were determined at weaning (postnatal day 21). Chi-square analysis was used to compare the observed values with the values predicted by Mendelian inheritance patterns. P < 10−3.
Increased rate of survival of FX(−/−) embryos in the progeny of FX(−/−) × FX(+/−) matings, and FX(+/−) intercrosses

Rare viable FX(−/−) progeny of the FX(+/−) matings were runted, and died shortly (2–3 wk) after weaning. In an attempt to rescue these rare FX(−/−) individuals, FX(−/−) progeny obtained from FX(+/−) mothers gestated on fucose-supplemented water were weaned to chow and water that were supplemented with fucose. Two such individuals, both males, survived to and thrived in adulthood on the fucose-supplemented diet. Reasoning that if these founders were fertile, mating with heterozygous females might increase the number of FX(−/−) embryos per uterus eligible for survival, and would in principle double the number of viable FX(−/−) individuals available for study. However, surprisingly, matings between FX(+/−) females and the two founder FX(−/−) males (reared from weaning on a fucose-supplemented diet) yielded an ~2:1 ratio of FX(+/−) and FX(−/−) genotypes among the progeny, with normal sized litters, and an equal distribution of sexes. This ratio was not appreciably altered by supplementing the maternal diet with fucose prior to and during gestation (Table IV). Furthermore, matings between FX(−/−) males and females, both reared on a fucose-supplemented diet, also yielded normal sized litters containing equal numbers of male and female FX(−/−) progeny. These observations, and the fact that the complex genealogy of the FX(−/−) founder line is characterized by variable contributions by the C57BL/6J and 129S2/SvPas genomes (Becker, 2002; unpublished data), are consistent with the possibility that one or more modifier loci contribute to survival of FX(−/−) animals (unpublished data), or that the intrauterine distribution of FX genotypes determines the relative survival potential of FX(−/−) embryos (see Discussion).

Fucose-dependent restoration of health, viability, and cellular fucosylation in FX(−/−) mice

FX(−/−) mice are small at weaning, but overcome this deficit by 6 wk of age when reared on a fucose-supplemented diet (Fig. 5 A). The fucose-supplemented diet yields a modest but reproducible suppression of weight gain in heterozygotes (Fig. 5 B) and in WT mice (unpublished data). Its cause is not known. Male and female FX(−/−) mice have normal life spans through the first 2 mo of life (Fig. 5 B), and have grossly normal overall general health when provided with a fucose-supplemented diet. By contrast, FX(−/−) mice reared on standard laboratory chow remain smaller that WT or fucose-fed FX(−/−) littermates (Fig. 5), and consistently suffer from a clearly evident diarrhea. Diarrhea does not occur in FX(−/−) mice reared from birth on a fucose-containing diet, but reversion to a standard chow diet leads to onset of diarrhea an average of 16 d later, and terminates within 9 d of resuming a fucose-supplemented diet. Diarrhea is associated with colonic histopathology characterized by a thickened mucosa, crypt destruction and distortion, crypt abscesses, regenerative crypt and surface epithelium, and a mixed inflammatory cell infiltrate within the lamina propria. This histopathology is reversed by restoration of fucose supplementation, and is characteristic of inflammatory bowel disease (Fig. 6; Allison et al., 1998). Diarrhea is accompanied by evidence for dehydration, characterized by a “tenting” of the animal’s skin and a 48% average increase in serum creatinine, which may account for their extreme lethargy and short lifespan. Diarrhea and abnormal colon histopathology are not characteristic of mice with deficiencies in Fuc-IV and/or Fuc-TVII (Homeister et al., 2001; unpublished data), indicating that selectin ligand deficiencies in these mice, and in the FX(−/−) mice (see below) do not, per se, account for inflammatory bowel disease syndrome in FX(−/−) animals.

Male and female FX(−/−) mice are infertile when reared in the absence of fucose. FX(−/−) males reared from birth on a fucose-supplemented diet and with documented fertility become completely infertile ~6 wk after transfer to a standard diet despite an apparently normal mating ability at this time. Fertility is restored within 4 wk after return to fucose supplementation. Fucose-dependent fertility in female FX(−/−) mice follows a similar pattern.
In FX(−/−) mice, restoration to health with a fucose-supplemented diet implies that a relative or complete deficiency of FX-dependent GDP–fucose synthesis and cellular fucosylation accounts for failure to thrive. Immunohistochemistry was used to determine if the glycans in the tissues of adult FX(−/−) mice reared in the absence of fucose are deficient in fucose modifications, and if fucose supplementation circumvents this defect. In the mouse liver, a site of synthesis of (1,6)-fucosylated glycans (Havenaar et al., 1998; Miyoshi et al., 1999), Aleuria aurantia agglutinin, a lectin highly specific for (1-6)-linked fucose, binds to the N-glycans in WT mice reared on a standard diet, but binds to the N-glycans in FX(−/−) mice only when they are reared on a fucose-supplemented diet (Fig. 7 A). In the high endothelial venules (HEVs) of peripheral lymph nodes, where O-linked (1,3)-fucosylated glycans contribute to the adhesive activities of L-selectin counter receptors (Maly et al., 1996), an L-selectin–IgM chimera binds to HEVs in WT mice reared on a standard diet, but binds to FX(−/−) HEVs only when their diet is supplemented with fucose (Fig. 7 B). These observations imply that the N- and O-linked glycans in FX(−/−) mice are deficient in fucose modification unless the salvage pathway is supplied with exogenous fucose.

**Conditional expression of neutrophil E- and P-selectin counter receptor activities in FX(−/−) mice**

Fucosylation on O-glycans (Wilkins et al., 1996) and N-glycans (Steegmaier et al., 1995) also provides a primary contribution to the adhesive activities of leukocyte E- and P-selectin counter receptors (Maly et al., 1996; Homeister et al., 2001). To examine the hematological consequences of the conditional fucosylation deficiency characteristic of the FX(−/−) mouse in this context, leukocyte selectin ligand activities were assessed in these mice as a function of fucose repletion. In FX(−/−) mice raised on a standard diet, flow cytometry analysis of blood neutrophils discloses an absence of E- or P-selectin ligand activities (while retaining expression of the P-selectin ligand PSGL-1; unpublished data) and profoundly defective fucosylation of their surface-localized N-glycans (while retaining expression of complex N-glycans detected with concanavalin A; unpublished data) (Fig. 8 A).
However, full restoration of neutrophil E- and P-selectin ligand activities and N-glycan fucosylation are observed in FX(-/-) mice provided with cage water or chow supplemented with fucose at levels used in efforts to rescue embryonic lethality (Fig. 8 A). When FX(-/-) mice raised on a standard diet are provided with water supplemented with 2.5 mM fucose 3 d before phlebotomy, modest restoration of P-selectin ligand activity and N-glycan fucosylation is observed, whereas E-selectin ligand activity remains apparently absent. Small amounts of E-selectin ligand activity first become detectable by flow cytometry in mice provided with 5 mM fucose in water, and are accompanied by increased expression of P-selectin ligand activity and N-glycan fucosylation, relative to levels observed with 2.5 mM supplementation. With 10 mM fucose supplementation, virtually normal expression of P-selectin ligand activity and N-glycan fucosylation are observed, whereas E-selectin ligand activity is not fully reconstituted. An assessment of the E- and P-selectin–mediated shear-dependent adhesion of these neutrophils also demonstrates a dissociation between the relative degree of restoration of E- and P-selectin counter receptor activities (Fig. 8 B). This more sensitive approach for detecting E- and P-selectin ligand activities (Homeister et al., 2001) also discloses residual E-selectin ligand activity at 2.5 mM fucose supplementation not evident in the flow cytometry analysis (Fig. 8 B).

Mechanisms remain to be defined to account for the apparent differential reconstitution of P-selectin ligand activity, relative to restoration E-selectin ligand activity, at certain low levels of fucose supplementation. These mechanisms are likely to be a complex function of the number and site(s) of the fucose moieties required to reconstitute P- and E-selectin ligand activities on specific E- and P-selectin counter receptors, and perhaps of the relative efficiencies with which these sites are fucosylated at any given GDP–fucose concentration. Regardless of mechanism, these observations suggest that cellular control of GDP–fucose availability has the potential to differentially regulate the functionality of distinct fucosylated cell surface glycans, as inferred for events involving CMP–sialic acid-dependent sialylation (Keppler et al., 1999) and UDP-galactose–dependent galactosylation (Toma et al., 1996). The faster and greater restoration of P-selectin ligand activity, relative to E-selectin ligand activity, in a LAD-II patient treated with fucose (Marquardt et al., 1999) also supports this notion. Together with the results obtained with the lectin and...
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L-selectin–IgM chimera staining (Fig. 7), these observations indicate that a virtually complete fucosylation deficiency extends to many organs in FX(-/-) mice, and can be reliably circumvented via the salvage pathway and exogenous fucose.

Conditional modulation of blood leukocytosis in FX(-/-) mice

Defects in the selectin receptor/counter receptor couple lead to deranged homeostasis in blood leukocyte number (Frenette et al., 1996; Maly et al., 1996; Robinson et al., 1999; Homeister et al., 2001). Although homeostasis is inferred to be a function both of selectin ligand-dependent clearance of leukocytes, and production of leukocytes by their marrow-derived progenitors (Johnson et al., 1995; Frenette et al., 1996; Levesque et al., 1999; Homeister et al., 2001), the relative contributions of these two processes, and the temporal range over which each process contributes are not understood. The conditional nature of the neutrophil selectin ligand defect in FX(-/-) mice, with the potential to rapidly induce or extinguish expression of selectin ligands, presented an opportunity to examine and better define the relative contributions of selectin ligand-dependent leukocyte clearance and leukocyte production.

FX(-/-) mice reared since weaning in the absence of fucose exhibit a substantial blood leukocytosis, accounted for by a 25-fold increase in neutrophils (Fig. 9 A). In these mice, E- and P-selectin ligand activities are not detected on peripheral blood leukocytes (Fig. 8). In FX(-/-) mice reared in the absence of fucose, the neutrophilia is reduced by roughly half within 8 h of oral fucose administration, to a level approximating that observed in mice deficient for fucosyltransferases VII and IV (Homeister et al., 2001). The rapid reduction in neutrophilia is precisely paralleled by the appearance in the blood of a population of neutrophils that are P-selectin ligand positive and that comprise a majority of circulating neutrophils (Fig. 9 B). Some of the circulating neutrophils also express E-selectin ligands at this time, though these are fractionally fewer than the P-selectin ligand positive neutrophils, in keeping with the observation that E-selectin ligand activity is less complete than P-selectin ligand activity when examined under limiting conditions of fucose supplementation (Fig. 8). 16 h after fucose repletion, the neutrophilia is further reduced, and E- and P-selectin ligands are now expressed on an equal and nearly normal fraction of these circulating cells. By 24 h, E- and P-selectin ligands are expressed on the majority of blood neutrophils, as observed in WT mice (Maly et al., 1996) or in FX(-/-) mice reared from birth on fucose (Fig. 9 B). Rapid restoration of the blood neutrophil count to a near normal level thus parallels restoration of E- and P-selectin ligand activities. When considered together with the inferred role for selectins and their ligands.

Figure 8. Control of granulocyte selectin ligand expression with exogenous fucose. FX(-/-) mice were reared on 5% fucose-containing chow until 8 wk of age and then removed from exogenous sources of fucose for 7 d. The mice were then reintroduced to cages containing standard chow, and water containing the indicated concentration of fucose, or to cages containing 5% fucose-supplemented chow. Mice were killed three days later, and their peripheral blood neutrophils were subjected to flow cytometry analysis (A), or to controlled detachment assays in a parallel plate flow chamber (B). Peripheral blood leukocytes isolated from WT mice reared on standard chow were analyzed in parallel. For flow cytometry (A), blood leukocytes were stained with anti-Gr-1, and E- or P-selectin-IgM chimeras (without EDTA, solid line; with EDTA, dotted line), or PSA (without 100 mM mannose, solid line; with mannose, dotted line) (Materials and methods). In parallel plate flow chamber assays (B) (Materials and methods), purified blood neutrophils were allowed to settle for 2 min onto the selectin-IgG–coated plate (P-selectin = 38 sites/μm²; E-selectin = 47 sites/μm²) at 1 g, and were subjected to step-wise increments in shear stress lasting 30 s each. Cells remaining after each 30-s interval were quantitated by video microscopy and plotted as the fraction of cells present following initial static attachment.
in determining the circulating half-life of a neutrophil (Johnson et al., 1995; Homeister et al., 2001), these observations imply that a major determinant of the neutrophil in FX(−/−) mice is a prolonged half-life, and that this half-life, and thus the number of circulating neutrophils, is restored by fucose-dependent reconstitution of selectin ligand expression.

Circulating selectin ligand-deficient neutrophils generated by the marrow prior to fucose supplementation may convert to selectin ligand positivity acutely after fucose repletion, as selectin ligand activity on FX(−/−) neutrophils is partially restored when incubated in vitro in fucose-supplemented media (Fig. 9 C). In the marrow, E- and P-selectin ligand expression on Gr-1–positive myeloid lineage cells plateaus within 16 h of fucose administration, reaching near normal levels, and slightly anticipating full restoration of E- and P-selectin ligand expression in the periphery (Fig. 9 D). These observations imply that a substantial fraction of the selectin-ligand positive neutrophils in the periphery represent cells newly formed by fucose-repleted progenitors in the marrow, and then released rapidly into the circulation. The order of restoration of E- and P-selectin ligands on marrow cells is opposite to that observed in the periphery, suggesting that some of these E-selectin ligand positive marrow cells may not reach the periphery.

The myeloid–erythroid (M/E) ratio in the marrows of FX(−/−) mice reared in the absence of fucose is increased by more than twofold (Fig. 9 E), and is accompanied by a nearly twofold increase in colony-forming unit–granulocyte macrophage (CFU-GM), the mature subsets of myeloid progenitor cells that respond to stimulation by GM–colony-stimulating factor or M-CSF (Fig. 9 E). The number of CFU-GM is reduced by half at 24 h, and normalizes by 48 h after fucose repletion, whereas the M/E ratio normalizes between 48 and 96 h later. To the extent that the lineage-committed progenitor cells represented by the CFU-GM pool correlate with the granulocyte production by the marrow, these observations imply that selectin ligand deficits, or deficits in other fucose-dependent processes in the marrow, are accompanied by aberrant proliferation of myeloid progenitors and an increased production of granulocytes that contributes to the neutrophilia. Normal numbers of burst-forming unit–erythroid and CFU–granulocyte-erythroid-megakaryocyte-macrophage are present in the marrows of the FX(−/−) mice, and do not vary as a function of fucose repletion (unpublished data), suggesting that defective fucosylation does not significantly perturb homeostasis of committed erythroid lineage progenitors, nor of more primitive multipotent progenitors.

In FX(−/−) mice a modest neutrophilia persists at 24 h after the initiation of fucose supplementation, remains for at least 4 d, but returns gradually to a normal level over a period of three or more weeks (unpublished data). Because selectin ligand expression has fully normalized by 24 h, and the CFU-GM and M/E ratio normalize within 96 h, these observations imply the existence of fucose-dependent processes that directly or indirectly determine blood neutrophil homeostasis, and that are not yet apparent from our analysis of the marrow or peripheral blood leukocytes.

The virtually normal neutrophil counts observed in FX(−/−) mice reared on a fucose-replete diet give way to a neutrophilia when these mice are fed a standard diet (Fig. 10 A). A modest neutrophilia is apparent within 3 d after termination of fucose supplementation, corresponding to the loss of selectin ligand-positive neutrophils (Fig. 10 B), and gradually increases over the ensuing 12 d. In the marrow, the number of CFU-GM remains normal during the first day after fucose withdrawal, then increases by 3 d to the roughly twofold elevation observed in chronic absence of fucosylation, in approximate correlation with an increase in the M/E ratio and onset of neutrophilia (Fig. 10 C). The more gradual onset of peripheral and marrow hematopoietic abnormalities upon fucose withdrawal may in part reflect the pace of replacement of fucosylated cellular and extracellular glycoproteins with nonfucosylated versions that no longer sustain hematopoietic homeostasis. During glycoprotein turnover, endocytosis (Mukherjee et al., 1997) and autophagy (Klonsky and Emr, 2000) deliver fucosylated glycoproteins and glycolipids to the lysosome, where lysosomal hydrolases that include α-fucosidase (Winchester, 1996) liberate fucose. Transport of this fucose into the cytosol (Jonas et al., 1990), and utilization by the salvage pathway, may prolong the pace of replacement with nonfucosylated glycans.

Discussion

Mutant cell lines with deficiency in GDP-mannose 4,6-dehydratase (GMD), the first enzyme in the de novo pathway (Fig. 1), do not elaborate fucosylated glycans (Reitman et al., 1980; Ripka et al., 1986). Quantitative studies in HeLa cells also indicate that the de novo, mannose-depended pathway accounts for the vast majority of cellular GDP–fucose production (Yurchenco and Atkinson, 1977). FX-deficient mammalian cell lines have not been previously described; however, as demonstrated here, targeted disruption of the FX locus in the mouse ablates the de novo pathway for GDP–fucose synthesis from GDP–mannose (Fig. 1), creating an animal that is entirely dependent on exogenously supplied fucose for cellular fucosylation. FX(−/−) adult animals exhibit a virtually complete absence of fucosylated glycans in multiple tissues that can be intentionally reversed by modulation of dietary fucose.

The situation is more complex in the embryo. Because fibroblasts derived from FX(−/−) embryos exhibit a complete deficiency in fucosylation unless supplied with exogenous fucose (Fig. 4), the existence of fucosylated glycans in FX(−/−) embryos gestated in FX(+/−) mothers fed standard laboratory chow suggests that fucosylation derives from maternal or fraternal sources. Though free serum fucose concentrations are not known in mice, micromolar quantities of nonprotein associated fucose are present in the serum of rats (Sima et al., 1997). Transplacental delivery of free fucose from maternal serum to FX(−/−) embryos, or from FX(+/−) or FX(+/+) siblings, perhaps via a specific plasma membrane transporter (Wiese et al., 1994), could supply the salvage pathway for GDP–fucose synthesis in developing embryos. FX(−/−) embryos may also be supplied with fucose via transcytotic or endocytotic delivery of maternal fucosylated glycoconjugates to the embryo, via the placenta. Fucose released from these molecules by lysosomal catabolism, and delivered to the cytosol by a lysosomal fucose transport sys-
Conditional control of fucosylation in FX-null mice

Jonas et al., 1990, could also supply the salvage pathway. The existence of maternal-fetal fucose transport remains to be established, and it is not known if systems like the placental IgG transport system (Mostov, 1994) can deliver meaningful amounts of maternally-derived fucosylated glycoconjugates to the catabolic machinery of the embryo. Our observations imply the existence of such pathways.

The embryonic lethal phenotype characteristic of FX(−/−) mice is unusual. Fucosylated glycans are widely expressed in the mouse embryo (Sato et al., 1986), have been assigned essential roles in implantation (Kimber, 2000), and blastocyst compaction (Fenderson et al., 1984), and their absence early in development might have been suspected to lead to adverse or lethal consequences. Mice that are homozygous for null alleles at loci encoding the (1,3)fucosyltransferase Fuc-TIV, Fuc-TVII, or both (Maly et al., 1996; Homeister et al., 2001), or in the (1,2)fucosyltransferases FUT1 or FUT2 (Domino et al., 2001) survive to term at a normal rate. The embryonic lethality of FX(−/−) progeny from an FX(+/−) intercross might therefore be ascribed to deficiencies in fucosylation events determined by (1,3)fucosyltransferase Fuc-TIX (Kudo et al., 1998), by (1,6)fucosyltransferase Fuc-TVIII (Miyoshi et al., 1999), by one or more serine/threonine fucosyltransferases (Harris and Spellman, 1993), or by candidate fucosyltransferases yet to be characterized (Roos et al., 2002).

However, fucosylated glycans are detected in FX(−/−) embryos generated from an intercross of FX(+/−) mice, a circumstance associated with highly penetrant embryonic lethality (Table I). Maternally derived fucose may restore cellular GDP–fucose to a level sufficient for appropriate fucosylation of some glycoproteins and glycolipids, but not others whose fucosylation may be essential for function, and perhaps embryonic viability. The fact that a low level of fucose supple-
mentation restores P- but not E-selectin ligand expression on neutrophils in the FX(+/H11002/H11002) mice, and the observation that fucose treatment of a LADII patient yields greater restoration of P-selectin binding relative to E-selectin binding (Marquardt et al., 1999) provide precedents for this possibility.

Alternatively, the GDP–fucose salvage pathway may not be active in all cell types, in utero or otherwise, leaving such cells, in FX(+/H11002/H11002) mice, vulnerable to a complete deficiency of fucosylation. We do not know if the salvage pathway is absent from some cells, or if there are cell types for which a complete defect in fucosylation will confer lethality upon an embryo. It seems unlikely that the product of the GMD protein, GDP-4-keto-6-deoxymannose (Sullivan et al., 1998) or its unknown metabolites which might accumulate in FX(+/H11002/H11002) embryos, are toxic and account for intrauterine demise, as FX(+/−) embryos appear to be produced in normal numbers in FX(+/−) intercrosses. Other fucosylation-independent mechanisms that might account for the lethal embryonic phenotype remain to be identified and tested.

The results of crosses between FX(−/−) and FX(+/−) mice (Table IV) and intercrosses of FX(−/−) mice indicate that parental genotype at the FX locus correlates with the penetrance of the embryonic lethality associated with nullizygosity at the FX locus. These data, and the derivation of the FX(−/−) colony from two surviving male FX(−/−) males, via a complex and interbred genealogy are also consistent with the possibility that segregation of one or more modifier alleles derived from either the C57BL/6J or 129/Sv ancestors of the FX(−/−) mice may suppress or authorize embryonic lethality when segregating with FX null alleles. This notion is strongly supported by our recent observation that matings between FX(+/−) mice obtained after 12 generations of backcrossing to the C57BL/6J strain yield FX(−/−) progeny at a nearly Mendelian rate (unpublished data). Isolation of such strain-specific modifier loci may prove instructive in understanding the contributions of fucosylated glycoconjugates to development and differentiation.

Parallels between the phenotype we observe in the FX(−/−) mice and those observed in LAD-II, a human disorder of fucosylation consequent to mutations in the Golgi GDP–fucose transporter locus (Lubke et al., 2001; Luhn et al., 2001), extend to the ability to correct the fucosylation defects in culture with fucose (Karsan et al., 1998), and for one LAD-II pedigree, restoration of leukocyte selectin ligands with oral fucose administration (Marquardt et al., 1999). FX deficiency and LAD-II are also characterized by a rather nonspecific growth deficiency, but it remains to be determined if the dysmorphology and psychomotor retardation characteristic of LAD-II have parallels in FX deficiency. However, colitis is not a prominent feature of LAD-II, and there is discordance between the embryonic lethality seen in FX(+/−) intercrosses and an apparent absence thereof in heterozygous matings within LAD-II pedigrees (Becker and Lowe, 1999). Lack of phenotypic overlap in these and other contexts may be ascribed to the fact that the FX(−/−) mice are completely dependent upon the salvage pathway for GDP–fucose availability, whereas residual GDP–fucose transport activity characteristic of the hypomorphic transporter defi-
ciencies is likely to support some level of fucosylation in utero and post-natally, with the caveat that it is not yet known if deficiencies at these two loci also yield fucosylation-independent events that contribute to phenotype.

Fucose modifies the EGF-like domains of some blood clotting proteins (Harris and Spellman, 1993) and the Notch family of transmembrane receptors (Moloney et al., 2000a) through the hydroxyls of serine and threonine residues. Members of the Fringe family of N-acetylglucosaminyltransferases require this fucose modification to construct a Notch-associated glycan that modulates signal transduction events initiated by Notch ligands (Bruckner et al., 2000; Moloney et al., 2000b). These considerations imply that Fringe-dependent Notch signaling will be perturbed in FX(−/−) mice, and suggest that conditional control of expression of this elongated glycan in these animals may be used to further define the roles for Fringe-dependent control of Notch signaling in lymphoid development (Deftos and Bevan, 2000), hematopoiesis (Milner and Bigas, 1999), epithelial cell differentiation (Lowell et al., 2000), vertebrate somitogenesis (Evard et al., 1998; Zhang and Gridley, 1998), and the general establishment of developmental boundaries during development (Irvine, 1999).

Materials and methods

Generation of targeted embryonic stem cell lines

A targeting vector constructed from the murine FX locus (Szkoka et al., 1990; Camardella et al., 1995) contains −2.45 and 2.10 Kb of the 5’ and 3’ ends, respectively, of the FX locus isolated from the 129/Sv mouse strain. D3 embryonic stem cells (129Sv/SePas; Simpson et al., 1997) were transfected, selected, and maintained as described (Maly et al., 1996). Correctly targeted embryonic stem cells were identified by Southern blot analysis using a 427-bp fragment amplified by the polymerase chain reaction (5’ primer: TTGCGGAGATAAGAAGTCTCCTCC; 3’ primer: AGACCACTGGACAGCTTCTAGG) from genomic DNA located external to the 5’ end of the targeting construct to probe EcoRI-digested genomic DNA (Probe A, Fig. 2A, 8.9-Kb WT allele, 3.2-Kb mutant allele). Correct homologous integration was confirmed by probing the same blot with a 1320-bp fragment amplified from genomic DNA located external to the 3’ end of the targeting construct (Probe B, Fig. 2A, 8.9-Kb WT allele, 4.3-Kb mutant allele; data not depicted). Deletion of exons 4 and 5 was confirmed by probing the same blot with a 453-bp Sac I-Msp I fragment (Probe C, Fig. 2A, 8.9-Kb WT allele). Second site nonhomologous integration events were excluded by probing Southern blots with a fragment isolated from the PGK-Neo cassette (Probe D, data not depicted).

Generation of FX(−/−) mice

FX(−/−) embryonic stem cells were injected in C57BL/6J blastocysts, followed by transfer into CD-1 pseudopregnant recipient females. Chimeric males were crossed with C57BL/6J females. The mutant allele was identified in F1 progeny by Southern blot analysis of EcoRI digested genomic DNA using probe A (Fig. 2A, 9.0-Kb WT allele, 5.7-Kb mutant allele; FX-null mice were established by mating FX(+/−) heterozygous mice supplemented with 100 mM fucose in sterile water ad libidum. FX(+/+) and FX(−/−) mice were maintained in a nude mouse vivarium at The University of Michigan (Ann Arbor, MI). Embryonic FX(−/−) mice were identified from the PGK-Neo cassette (Probe D, data not depicted). Deletion of exons 4 and 5 was confirmed by probing the same blot with a 1040-bp Sac I-Msp I fragment (Fig. 2A, 8.9-Kb WT allele). Second site nonhomologous integration events were excluded by probing Southern blots with a fragment isolated from the PGK-Neo cassette (Probe D, data not depicted).

Fucose supplementation

Mice were maintained on either Modified Lab Chow (product #5723C-A; Purina Mills Diet) containing 5% (matine) or 0.5% (maintenance) (wt/wt) fucose (Pfanstiehl), or sterile water containing 100 mM fucose, or both, supplied ad libidum. To assess leukocyte selectin ligand expression and blood leukocyte counts after acute fucose repletion, 200 μl of 0.5 M fucose in endotoxin-free normal saline was administered by tail vein injection, and the mouse was immediately placed into a cage with water supplemented to 100 mM fucose.

Flow cytometry of embryonic fibroblasts

Embryonic fibroblasts were prepared from 14.5 dpc embryos as described (Robertson, 1987), and were incubated at 37°C in DME (Bio-Whittaker) containing 5% fetal bovine serum, ± 22 μM fucose. Cells were stained with PSA-FITC (EY Laboratories; 1:600) and analyzed as described (Maly et al., 1996).

Enumeration and analysis of blood and marrow leukocytes

Collection and flow cytometry analysis of selectin ligands on blood leukocytes has been described (Maly et al., 1996). FITC-conjugated PSA (1:1,000) and concanavalin A (1:50) were used ± mannose (100 mM) or α-methylmannoside (200 mM), respectively. Colony forming units in the marrow were determined with a commercially available methylcellulose-based procedure (StemCell Technologies, Inc.). Assay of controlled leukocyte detachment in a parallel plate flow chamber was done as described previously (Homeister et al., 2001). Restoration of neutrophil fucosylation in vitro was done by incubating blood leukocytes for 8 h in DME containing 10% fetal calf serum ± 22 μM fucose, followed by flow cytometry analysis.

Immunohistochemistry

Detection of HEV-born L-selectin ligands has been described (Maly et al., 1996). Digoxigenin-labeled Aleuria aurantia lectin (5 μg/ml; Roche) (Yamashita et al., 1985) was used to stain frozen sections of liver. Digoxigenin-labeled lectin was visualized with peroxidase labeled anti-digoxigenin antibody (1:50 units/ml; Roche). Spleens were examined in WT or FX(−/−) embryos obtained at 18.5 dpc from an FX(+/−) mother sired by an FX(+/+) male, and gestated without fucose supplementation. Frozen sections (10 μm) was air dried, blocked with 2% BSA in TBS, pH 7.4, 2 mM CaCl2, and 0.02% azide, and stained with an E-selectin/FGM chimera (Maly et al., 1996) ± 5 mM EDTA. The sections were washed with TBS/2 mM CaCl2 or TBS/5 mM EDTA, and then incubated with biotin-conjugated goat anti-human IgM followed by FITC-conjugated streptavidin (Pharmingen). Parallel sections were stained with Gr-1 biotin-conjugated anti-Gr-1 (1:200; Pharmingen), and Texas red–conjugated streptavidin (1:200; GIBCO BRL). Sections were washed with TBS/2 mM CaCl2, fixed with 10% formalin, washed with PBS, mounted with Citifluor AF1, and examined by fluorescence microscopy.

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