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Glycosylation Changes as Markers for the Diagnosis and Treatment of Human Disease

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Introduction

Carbohydrates are critical components of every living organism; they occur in many diverse forms and fulfill numerous biological roles. Collectively, they provide energy for cellular metabolism, mediate intracellular signalling and extracellular communication events, and constitute major structural components of the extracellular matrix. Considering their importance, it is not surprising that carbohydrate-related metabolic abnormalities, especially those that are linked to oligosaccharide biosynthesis, result in human disease. Over the past three decades, an impressive compilation of disease-associated changes to oligosaccharide structure, as well as defects in the metabolic pathways responsible for the biosynthesis of these structurally diverse and informationally rich molecules, has become available. One goal of this report is to survey representative glycosylation changes and describe how these 'markers' are exploited for the detection and diagnosis of a growing number of diseases. Knowledge of the genetic or structural basis of a glycosylation defect implicated in a disease facilitates clinical diagnosis, but is often of minimal therapeutic value. A second goal

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Abbrevations: AGE, advanced glycation endproducts; APP, amyloid precursor protein; CDG, congenital disorder of glycosylation; Dol-P, dolichylpyrophophosphate; ER, endoplasmic reticulum; ESI-MS, electrospray ionization mass spectrometry; Fructose-6-P, fructose-6-phosphate; GalCer, glycolipid galactosylceramide; GlcNAc, *N*-acetylglucosamine; GlcNAc-T, *N*-acetylglucosaminyltransferase; GlcNAc-TV, *N*-acetylglucosaminyltransferase V; HA, haemagglutanin; HIBM, hereditary inclusion body myopathy; HIV, human immunodeficiency virus; IEF, isoelectric focusing analysis; IgAN, immunoglobulin A nephropathy; ISSD, infantile sialic acid storage disease; LDL, low-density lipoprotein; LLO, lipid-linked oligosaccharide; LPS, lipopolysaccharides; MAbs, monoclonal antibodies; Man, mannose; Man-6-P, mannose-6-phosphate; ManNAc, *N*-acetylmannosamine; MC, mesanglial cell; MDM, monocyte-derived macrophages; NA, neuraminidase; NCAM, neural cell adhesion molecule; OST, oligosaccharyltransferase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; Tf, transferrin.

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of this report, therefore, is to outline how the partnership between physicians working in the clinic and glycobiologists working at a basic research level is currently undergoing a transition from a largely *descriptive* endeavour designed to identify disease markers to now focus on actively exploiting carbohydrates for the *treatment* of disease.

Inherited disorders and cancer supply two examples of why it is necessary to gain a *functional* understanding of how a glycosylation defect causes a disease to fully exploit carbohydrates as medicines. First, many specific genetic defects that impact the 'glycosylation machinery' of a cell have been identified as causative factors in a number of congenital diseases. Consideration of these molecular (most often enzymatic) defects in isolation, however, rarely accounts for the disease phenotype in a satisfying manner, as patients with the same genetic abnormality can experience widely divergent clinical symptoms (Freeze, 2001). Diversity in the manifestation of disease is the result of the genetic, proteomic, and environmental background of the affected cell, tissue, or patient, and unravelling the complex combination of interacting factors involved is an important step towards the development of a full contingent of treatment options.

Altered patterns of glycosylation that are ubiquitously associated with cancer provide a second example of how the present understanding of glycosylation markers is inadequate for effective treatment of disease. The identification of tumour-associated oligosaccharide structures has provided a set of extraordinarily useful markers for diagnostic, and even prognostic, purposes; however, it is now clear that the aberrant carbohydrate structures themselves play an active role in the development of the cancer disease phenotype. Consequently, an ability to repair defective carbohydrate components of a cell would hold considerable therapeutic value. However, unlike congenital disorders, the biosynthetic defects responsible for cancer-associated glycosylation abnormalities frequently remain unknown at a genetic or proteomic level, thereby precluding any rational attempts to fix the glycosylation machinery. In addition to congenital disorders and cancer, aberrant glycosylation also occurs in many other important human ailments, such as diabetes and atherosclerosis, as well as neurodegenerative and immune disorders; several of these diseases will be outlined in this report to provide an overview of the diversity of glycosylation changes that impact human health. Finally, although this report focuses on *abnormalities* that result in disease, it is worth noting that carbohydrate-related disease can also occur in the absence of glycosylation defects. In these cases, pathogens exploit *naturally occurring* carbohydrates as binding epitopes to achieve uptake and colonization of a cell.

A thorough understanding of carbohydrate metabolism at a cellular level is a valuable and necessary asset for an investigator seeking to identify specific glycosylation changes implicated in disease, assign the exact role of the defect in disease etiology, and develop treatment options. First, a description of the glycosylation machinery of a healthy cell is needed so that putative defects can be identified as harmless polymorphisms, or recognized as sinister aberrations with disease implications. Next, a step-by-step description of how glycosylation changes at a molecular level translate into the symptoms observed in patients must be established. This process includes identification of the defective biosynthetic enzyme (or other protein), isolation, and structural characterization of the resultant altered oligosaccharide,

description of how the defective biosynthetic pathway and its products interact with other cellular players to disrupt cellular physiology, and, finally, a mechanistic explanation of how cell and tissue changes trigger clinical symptoms in a patient. While useful diagnostic, and even therapeutic, advances have emerged from 'bits and pieces' already known, having the 'big picture' in hand promises to identify numerous additional therapeutic targets, and will greatly advance the medicinal exploitation of carbohydrates.

Overview of carbohydrate metabolism and cellular glycosylation

In recent years, dozens of the enzymes, transporters, and accessory proteins that comprise the 'glycosylation machinery' of a cell have been cloned and studied in detail by classical biochemical methods. Hundreds of other protein components of this system have been tentatively identified by genomic methods, but their exact functional contributions remain to be defined in detail. In addition to their genetic constituents, the biosynthetic networks responsible for glycosylation also consist of small molecule intermediates that include monosaccharides and necessary co-substrates required for the progressive assembly of large carbohydrate structures. While enzyme-by-enzyme and intermediate-by-intermediate analysis has already yielded a robust picture of cellular carbohydrate metabolism (*Figure 10.1* and descriptions below), rapid discovery made possible by emerging genomic, proteomic, and 'glycomic' methods will facilitate the continued development of an increasingly finely textured portrait of the intricacies of these complex, interconnected metabolic networks.

An outline of overall cellular carbohydrate metabolism is shown in *Figure 10.1*. For the purposes of the present discussion, this process is considered to 'begin' when dietary sugars, after digestive reduction to their monosaccharide components, are taken up by the cell (*Figure 10.1*, Step 1). Once inside a cell, monosaccharides undergo chemical conversions that include stereochemical inversions, acetylation, phosphorylation, and dephosphorylation (Step 2). These processing reactions (Goon and Bertozzi, 2001) provide monosaccharide-derived intermediates needed for energy production (Step 2a, glycolysis), and also supply sugar nucleotide 'building blocks' (Step 2b) that are consumed during oligosaccharide biosynthesis (Step 3). Typically, complex carbohydrates are not produced as free-standing molecules, rather they are assembled while attached to either a lipid or protein scaffold; these composite structures are referred to as 'glycolipids' and 'glycoproteins', respectively (or, generically, as 'glycans'). Glycoproteins can be further divided into two major categories: *O*-linked, where the carbohydrate moiety is attached to either a serine or threonine residue (van den Steen *et al.*, 1998), or *N*-linked, where the carbohydrate is attached to an asparagine residue (Roth, 2002). Glycolipids and *O*-linked glycoproteins are synthesized *de novo* through the step-by-step addition of monosaccharide units. By contrast, synthesis of *N*-linked glycoproteins proceeds by the *en bloc* transfer of a 17-residue core lipid-linked oligosaccharide (LLO, *Figure 10.2*) to the recipient protein (Burda and Aebi, 1999). The final structure of the *N*-linked glycoprotein is specified by subsequent glycosidase trimming and further elaboration by glycosyltransferases (Fukuda, 2000). After synthesis is complete, relatively small *oligo*saccharides are transported to the cell surface (Step 4a), or are secreted (Step 4b)

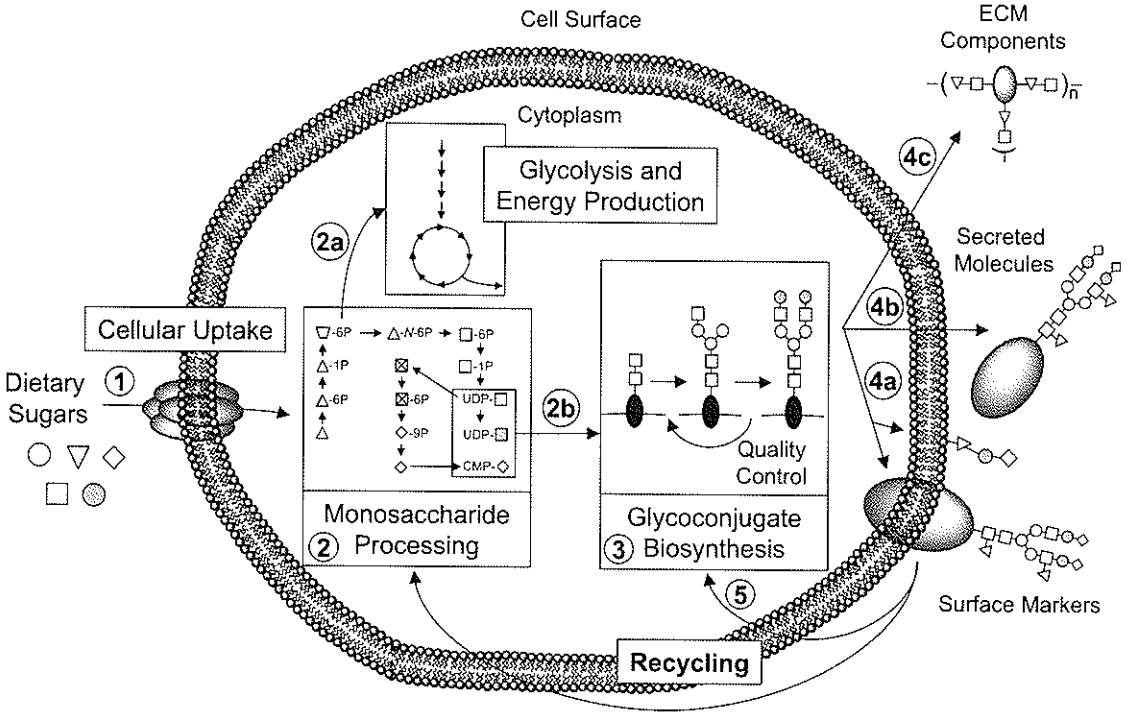

Figure 10.1. Schematic outline of cellular carbohydrate metabolism. This process begins with the (1) uptake of exogenous monosaccharides into the cell, where they undergo processing reactions (2) that convert the sugar into the appropriate intermediary forms required for glycolysis and the TCA cycle (2a) or glycoconjugate biosynthesis (3). Upon reaching maturity, glycoproteins and glycolipids are transported to the plasma membrane, where they function as surface markers (4a), are secreted from the cell (4b), or exported to become constituents of the extracellular matrix (4c). Finally, surface (and other exogenous) carbohydrates can be recycled and reused (5). The CDGs discussed in this report occur at the interface of Steps 2b and 3, and are often diagnosed by the analysis of secreted transferrin (4b). Cancer is marked by the aberrant display of oligosaccharide-based surface markers (4a and *Figure 10.2*); other diseases result from defects spread across the carbohydrate metabolic spectrum. It should be noted that each of these 'steps' involves the complex interplay of dozens (or hundreds) of structural elements and functional components; a fuller description of each is available from the references cited in the text.

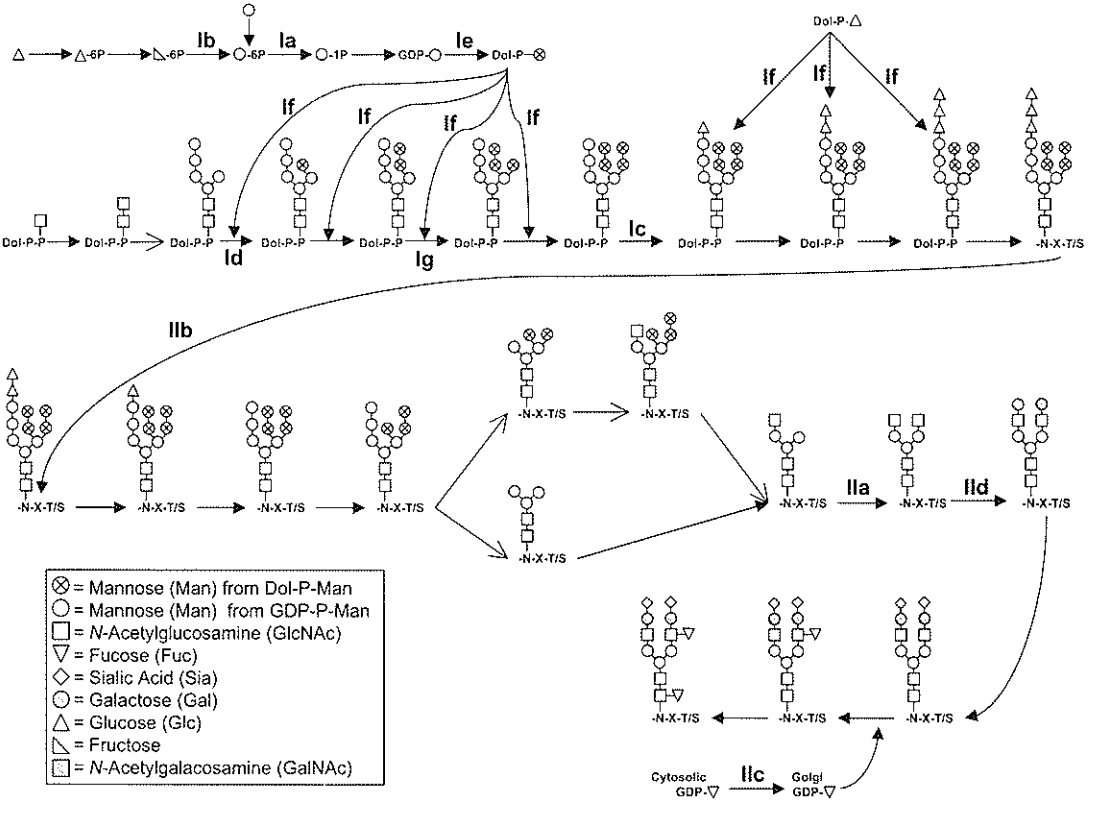

Figure 10.2. Outline of *N*-linked glycoprotein biosynthesis and the location of molecular defects implicated in congenital disorders of glycosylation (CDGs). Inherited disorders denoted by formal 'CDG' nomenclature impinge on the production of lipid-linked oligosaccharide (LLO), transfer of its carbohydrate moiety to the asparagine residue of the (-N-X-T/S-) consensus sequence within a nascent protein, or subsequent oligosaccharide trimming and re-elaboration. The location of each currently known CDGs, Type Ia–g, and Type IIa–d, are indicated. Closed arrows depict a single step reaction, open arrows represent a multi-step process, and Dol-P and Dol-P-P indicate dolichol phosphate and dolichol pyrophosphate, respectively. This figure is adapted and updated from Freeze (2001).

and serve to mediate cell–cell communication and signalling. Similarly, much larger *poly*saccharides are exported from the cell and become major constituents of the extracellular matrix (Step 4c). Finally, to complete the picture, cell surface carbohydrates are recycled into endocytotic vesicles, digested to their constituent monosaccharides, and reused (Step 5).

Congenital disorders of glycosylation (CDGs)

An ever-growing number of congenital diseases are associated with specific molecular defects that lurk within the metabolic pathways responsible for glycoconjugate biosynthesis. Up to now, a preponderance of clinically observed disorders, formally termed 'congenital disorders of glycosylation' (CDGs), has been traced to defects within the *N*-linked glycoprotein biosynthetic process (*Figure 10.2*). CDGs are classified into Type I and Type II disorders. Approximately 30 enzymes are currently known to participate in the pre-Golgi (Type I) steps, and at least 20 more in the Golgi-localized (Type II) processing reactions (Freeze, 2001); so far, defects in 11 different genes that lead to human disease have been identified. Clinical symptoms, genetic and molecular defects, and treatment options, when available, are outlined below.

TYPE I CDGs

Type I CDGs are linked to defects in monosaccharide processing (Step 2b of *Figure 10.1*) and oligosaccharide biosynthesis (Step 3) that are spatially located in either the cytoplasm or endoplasmic reticulum (ER). These pre-Golgi disorders specifically alter the synthesis and transfer of the dolichylpyrophophosphate-linked precursor oligosaccharide (or lipid-linked oligosaccharide; LLO) onto the nascent protein. Currently, known Type I disorders are specified as Ib, Ia, Ie, If, Id, Ic, and Ig, and are briefly described in the order that they occur in the metabolic pathway (by convention, letters are assigned chronologically in the order of discovery, not by their position in the metabolic pathway, *Figure 10.2*).

CDG Ib

The CDG Ib clinical phenotype, observed in approximately 20 known cases, is characterized by gastrointestinal disorders and protein-losing enteropathy, but does not include mental retardation or neuropathy (Niehues *et al.*, 1998). Patients experience a Man-6-P deficiency due to mutations in the *PMI1* gene that affect the ability of the phosphomannose isomerase (PMI) enzyme to convert fructose-6-phosphate (Fructose-6-P) into mannose-6-phosphate (Man-6-P) (Proudfoot *et al.*, 1994; Schultz *et al.*, 1994). Because the liver and intestine consume the most mannose, these organs are the most adversely affected by a mannose deficiency; a common biochemical feature in PMI-deficient patients is hypoglycosylation of liver-derived serum glycoproteins.

Man-6-P can be obtained from either fructose-6-P metabolism or from external sources; a normal diet, however, does not provide sufficient mannose to meet the requirements of glycoprotein biosynthesis in the liver in CDG Ib patients. If supplied exogenously, free mannose can enter the cell by a specific transporter and be

converted to Man-6-P by hexokinase, thereby bypassing the CDG Ib deficiency (Westphal *et al.*, 2001b), allowing patients to respond positively to mannose administered through the gut. Mannose therapy is particularly successful in young patients (Babovik-Vuksanovic *et al.*, 1999; de Lonlay *et al.*, 2001). This treatment, however, does not completely restore normal levels of overall glycosylation to serum proteins (Rush *et al.*, 2000; Westphal *et al.*, 2001b).

CDG Ia

Patients afflicted by CDG Ia, the most common CDG, experience cerebellar hypoplasia, liver dysfunction, ataxia, abnormal eye movements, variable mental retardation, and coagulopathy (Stibler *et al.*, 1998; Matthijs *et al.*, 2000). Mortality rates approach 20% in the first few years of life due to severe infections, liver insufficiency, or cardiomyopathy (Matthijs *et al.*, 2000). Variation in the clinically observed phenotype is linked to the exact mutation responsible for causing CDG Ia (van Ommen *et al.*, 2000; de Lonlay *et al.*, 2001; Leonard *et al.*, 2001).

Fibroblasts from CDG Ia patients synthesize truncated LLO, featuring four or five Man residues in lieu of the normal nine. This abnormality is caused by a deficiency in phosphomannomutase (PMM); so far over 60 different mutations in the *PMM2* gene have been linked to this disorder. PMM mediates the conversion of Man-6-P to Man-1-P; Man-1-P is then converted to GDP-Man; mutations in the *PMM2* gene decrease the catalytic activity of the resultant enzyme, or affect its stability, resulting in decreased manufacture of Man-1-P and, subsequently, GDP-Man (Korner *et al.*, 1998; Pirard *et al.*, 1999; Westphal *et al.*, 2001a). CDG Ia levels of GDP-Man are approximately ten times higher than levels found in normal cells (Pannerselvam and Freeze, 1996) and, because GDP-Man is necessary for *N*-linked glycan synthesis and GPI-anchors (Matthijs *et al.*, 2000), it is not surprising that truncated forms of LLO are produced in these patients. Standard diagnostic procedures cannot distinguish CDG Type Ia from CDG Type Ib. The only way to differentiate these disorders is by the response of a patient to mannose therapy. As mentioned above, treatment of CDG Ib has shown some success; by contrast, treatment of CDG Ia with oral mannose has yielded no biochemical or clinical benefit (Kajergaard *et al.*, 1998; Mayatepek and Kohlmuller, 1998). However, modified derivatives of Man-1-P, such as nontoxic acetoxymethyl esters, may offer another possibility for therapy (Schultz *et al.*, 1993, 1994).

CDG Ie

Psychomotor retardation and hypotonia characterize the clinical phenotype of CDG Type Ie, a disease caused by the aberrant conversion of DGP-Man to Dol-P-Man. A mutation in the *DPM1* gene alters normal GDP-Man:Dol-P mannosyltransferase activity (Imbach *et al.*, 2000b; Kim *et al.*, 2000). Dol-P-Man, in turn, serves as a donor substrate in *N*-linked protein glycosylation; a Dol-P-Man deficiency thus alters the biosynthesis of glycophospholipid anchors (Maeda *et al.*, 1998; Tomita *et al.*, 1998), C-mannosylated proteins (Krieg, 1997, 1998), and possibly *O*-mannose-based oligosaccharides in neural tissues (Chiba *et al.*, 1997; Yuen *et al.*, 1997). Decreased Dol-P-Man activity is confirmed by a documented shortage of CD59 protein levels in

patients (Imbach *et al.*, 2000b). Despite the mutation, some residual enzymatic activity occurs, and the extent of this activity strongly affects a patient's clinical phenotype. Mannose therapy has shown conflicting results in Ie patients. In one study, Dol-P-Man activity increased with mannose supplementation, but it was unclear whether the administered mannose was the cause (Kim *et al.*, 2000); no effect was observed in another study, and it remains unclear whether supplementation therapy is beneficial (Imbach *et al.*, 2000b).

CDG If

CDG If is characterized by psychomotor retardation, seizures, gastrointestinal problems, dry skin and scaling with erythroderma, hypoglycosylated serum glycoproteins, and impaired vision (Kranz *et al.*, 2001). A mutation in the gene *MPDU1* is responsible for CDG If. The *MPDU1* gene product mediates the efficiency and bioavailability (but not the actual biosynthesis) of donor substrates for lipid-linked oligosaccharides. By a mechanism that remains unclear, this defect renders monosaccharide donors, Dol-P-Man and Dol-P-Glc, less available within the lumen of the rough endoplasmic reticulum. As a result, several intermediary oligosaccharides accumulate, reducing the production of mature $Glc_3Man_9GlcNAc_2$ LLOs (Hirschberg and Snider, 1987; Schenck *et al.*, 2001). Because the complete oligosaccharide is the preferred substrate for oligosaccharyltransferase (OST), OST activity, responsible for the transfer of the carbohydrate from the lipid carrier to a protein (Silberstein and Gilmore, 1996; Yan and Lennarz, 1999), is significantly impacted. It is believed that this defect may also affect GPI anchor biosynthesis and C-mannosylation, in addition to *N*-glycosylation (Kranz *et al.*, 2001), thereby impacting the severity of the Type If phenotype. This CDG can be treated with mannose, and possibly with externally administered GDP-Man, to correct defective mannosylation (Camp *et al.*, 1993). However, these approaches must be used with caution because they adversely affect glucosylation (Kranz *et al.*, 2001).

CDG Id

CDG Id patients are afflicted by severe neurological abnormalities, including hypotonia, severe psychomotor retardation, and seizures. Optic atrophy, associated with coloboma of the iris, is also seen. CDG Type Id, also known as CDG Type IV, is caused by a deficiency in Dol-P-Man:Dol-PP-$Man_5GlcNAc_2$ α-1,3-mannosyltransferase (α-1,3-Man-T), due to a mutation in the *ALG3* gene (Korner *et al.*, 1999a; Imbach *et al.*, 2000a). This enzyme transfers Man from Dol-P-Man to Dol-PP-$Man_5GlcNAc_2$ to form $Man_6GlcNAc_2$; transferase deficiencies lead to accumulations of Dol-PP-$Man_5GlcNAc_2$. The nature of this disorder (reduced, but not otherwise errant, enzymatic activity), allows a proportion of the expected proteins to be normally glycosylated.

CDG Ic

CDG Ic is the second most common CDG after CDG Ia. CDG Ic patients exhibit characteristics similar to those observed in mild PMM-deficiency (CDG Ia). They are

neurologically affected, mentally retarded and seizure-prone, they can lack cerebellar hypoplasia, have a dysmorphic appearance and strabismus (Freeze, 2001), and sometimes display muscular hypotonia and delayed statomotor development (Kim *et al.*, 2000; Matthijs *et al.*, 2000; Westphal *et al.*, 2000a,b). Eight different mutations associated with CDG Ic have been identified in the *ALG6* gene, which decrease α-1,3-glucosyltransferase activity and lead to the hypoglycosylation of proteins (Imbach *et al.*, 1999). This enzyme is responsible for adding the first glucose to Dol-PP-Man, the LLO precursor to *N*-glycosylation. In CDG Type Ic, the oligosaccharide precursor is either incompletely synthesized or inefficiently transferred; nonglucosylated oligosaccharides are poor receptors for the oligosaccharyltransferase, resulting in unglycosylated nascent protein sites (Murphy and Spiro, 1981; Burda and Aebi, 1998). Depending on the particular mutation impacting *ALG6*, the nonglucosylated form of the precursor oligosaccharide can vary from 20 to 90% (Imbach *et al.*, 2000a).

CDG Ig

In the newly discovered CDG Ig, patients are characterized by hypocalcaemia, psychomotor retardation, hypotonia, susceptibility to infection, progressive microcephaly, facial dysmorphisms, and convulsions. These symptoms arise from a mutation in the *ALG12* gene, resulting in a Dol-PP-Man:$Man_7GlcNAc_2$-PP-Dol α-1,6-mannosyltransferase deficiency. In yeast, the *ALG12* gene product is responsible for catalyzing the addition of the α-1,6-mannosyl residue to Dol-PP-$Man_7GlcNAc_2$; mutations found in CDG Ig patients reduce the ability of this enzyme to add the eighth mannose residue onto the LLO precursor. In fibroblasts with this defect, $Man_7GlcNAc_2$ accumulates until it constitutes about 95% of all LLO species, with the resultant reduction in the level of complete *N*-glycan side chains (Hansske *et al.*, 2002; Thiel *et al.*, 2002).

TYPE II CDG DEFECTS (OLIGOSACCHARIDE BIOSYNTHETIC DISORDERS)

Type II CDG defects impinge on oligosaccharide biosynthesis in the Golgi complex (Step 3 of *Figure 10.1*). These Golgi-localized disorders affect the trimming and subsequent elongation of the core oligosaccharide after transfer onto its recipient protein, and impact the trimming and elongation of *N*-linked oligosaccharides (Imbach *et al.*, 1999). Analogous to Type I CDGs, these disorders will now be discussed in the order of their occurrence in the metabolic pathway (IIb, IIa, IId and IIc; *Figure 10.2*).

CDG IIa

Type IIa was the earliest identified CDG; patients with this disorder experience severe psychomotor retardation, hypotonia, facial dysmorphy, a susceptibility to infections (Coddeville *et al.*, 1998), skeletal abnormality, and often, gastrointestinal complications. CDG IIa is caused by a mutation in the gene *MGAT2*. This mutation decreases the amount of UDP-GlcNAc:α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II (GlcNAc-TII) by up to 98% (Jaeken *et al.*, 1994, 1996b; Schachter, 2000). Monosialylated chains follow normal synthesis and transfer of LLOs due to an absence of the GlcNAc-β-1,2-Man-α-1,6 antenna (Freeze, 2001).

Fully sialylated transferrin (Tf) is absent in these patients; instead, a large amount of disialylated Tf is observed that arises from the joining of two monosialylated truncated chains (Coddeville *et al.*, 1998). In addition, levels of certain serum glycoproteins, including thyroxine-binding globulin, clotting factor XI, antithrombin III, proteins C and S, are decreased, whereas elevated levels of serum IgM and cerebrospinal fluid are present (Lacey *et al.*, 2001). In Mgat2 mice, haemorrhaging and abnormal microbial colonization occur, and can lead to high mortality during gestation and shortly after birth; a similar response in humans with Type IIa CDG likely results in a significant underestimation of the occurrence of this disease (Patterson, 1999).

CDG IIb

CDG IIb is caused by a deficiency in glucosidase I due to a mutation in the *GCS1* gene. Glucosidase I is responsible for removing the terminal α-1,2-glucose residue from the $Glc_3Man_9GlcNAc_2$-oligosaccharide after it is transferred to protein (de Praeter *et al.*, 2000). This disease is extremely rare; only one patient has been reported. This patient was characterized by hypotonia, seizures, hepatic fibrosis, and dysmorphic features. An interesting feature was that the patient's serum transferrin and β-cerebral spinal fluid proteins were both normal; diagnosis was based on the detection of abnormal urinary tetrasaccharide Glc_3Man.

CDG IIc

CDG IIc, also known as leukocyte adhesion deficiency Type II, is characterized by increased leukocyte counts in patients (Etzioni *et al.*, 2002). Patients exhibit mental retardation, short stature, severe psychomotor and growth retardation, and immunodeficiency, and the rare Bombay blood group (Etzioni *et al.*, 1992; Frydman *et al.*, 1992; Strula *et al.*, 2001). This disorder is attributed to a deficiency in GDP-fucose, a nucleotide sugar donor necessary for fucosylation of growing oligosaccharides after its importation into the Golgi from the cytosol. Because fucosyltranferase levels (Shechter *et al.*, 1995) and GDP-L-fucose synthesis can be normal (Korner *et al.*, 1999b), it is thought that the disease impairs the general utilization of GDP-L-fucose, instead of impacting specific fucosyltransferases. The resulting absence of fucosylated glycans on the cell surface adversely affects the expression of sialyl-Lewis^x antigen and H-antigen on neutrophils, which in turn contributes to faulty leukocyte adhesion (Etzioni *et al.*, 1992). One patient has responded favourably to fucose supplementation (Marquardt *et al.*, 1999) by regaining fucosylated glycans on leukocyte surfaces, as well as experiencing cessation of infection episodes, and psychomotor improvement. However, fucose did not restore all fucosylation, and supplementation in two other patients had little effect.

CDG IId

Clinical features of CDG IId are marked by progressive hydrocephalus, hypotonia, myopathy, mental and statomotor retardation, brain malformation, and blood clotting defects (Peters *et al.*, 2001). Aside from myopathy, clinical characteristics are similar

to those found in CDG Ia. CDG IId is due to a deficiency in UDP-Gal:*N*-acetylglucosamine-β-1,4-galactrosyltransferase I (β4GalT I), this enzyme is responsible for transfer of galactose from UDP-galactose onto terminal *N*-acetylglucosamine residues of complex oligosaccharides. In CDG IId patients, truncated β4GalT I that lacks enzymatic activity is produced (Hansske *et al.*, 2002). This loss of β4GalT I activity subsequently results in a general reduction in the oligosaccharide size due to a lack of sialic acid and galactose residues in *N*-glycans found in serum transferrin. Residual galactosylation at about 9% of normal levels is observed in some patients, and is likely due to the compensatory activity of other galactosyltransferases (Hansske *et al.*, 2002).

NON-CDG CONGENITAL DEFECTS IN CARBOHYDRATE METABOLISM

Glycosylation defects occur throughout the carbohydrate metabolic processes of a cell

Molecular defects that contribute to the disorders formally classified as CDGs occur at the interface of Steps 2b and 3 in the overall picture of carbohydrate metabolism presented in *Figure 10.1*. These disorders are closely linked to the specific process of the transfer of the carbohydrate moiety of LLO to an asparagine residue of a nascent protein, and likely involve less than 10% of the cellular complement of glycosylation-related genes. Although many cellular defects that lead to abnormal *N*-glycosylation of proteins likely remain to be discovered, four additional primary disorders have been described already: 1) mucolipidosis II (I-cell disease) and mucolipidosis III (pseudo-Hurler polydystrophy) (Reitman *et al.*, 1981); 2) Type II leukocyte adhesion deficiency (Etzioni *et al.*, 1992); 3) Type II congenital dyserythropoietic anaemia (HEMPAS disease) (Fukuda, 1990); and 4) paroxysmal nocturnal haemoglobinuria (Rosse, 1997). Additional oligosaccharide biosynthetic defects, beside the *N*-linked glycosylation defects, are likely to emerge in the genes specifically devoted to *O*-linked oligosaccharide, proteoglycan, and glycosylphosphatidylinositol anchor biosynthesis, as well as in phosphoglycosylation and C-mannosylation processing (Spiro, 2002). Other congenital disorders, although not formally involving oligosaccharide biosynthesis, can also impact glycosylation, and have been linked to disease. Specific examples of monosaccharide processing and recycling disorders that are linked to disease are given below.

Monosaccharide processing and recycling disorders in the sialic acid pathway

Several of the CDG Type I disorders already discussed involve the biosynthesis and processing of the nucleotide sugar 'building blocks' required for *N*-glycan assembly (*Figure 10.1*, Step 2b). Additional monosaccharide processing disorders have now been discovered; to give an example, defects in the gene encoding UDP-GlcNAc 2-epimerase/ManNAc 6-kinase cause two fundamentally different diseases. The first defect, found in sialuria patients, renders this enzyme overactive and results in highly elevated levels of sialic acid in the cytoplasm of fibroblasts and in the urine (Seppala *et al.*, 1991). Conversely, mutations thought to decrease the activity of this enzyme

are linked with hereditary inclusion body myopathy (HIBM) (Eisenberg *et al.*, 2001). A handful of diseases are also associated with the recycling and reuse of cell surface glycoconjugates (*Figure 10.1*, Step 5). Typically, recycling proceeds by encapsulation of a glycan into a lysosomal vesicle where glycosidases liberate monosaccharides. Galactosialidosis (de Geest *et al.*, 2002) and sialidosis (described in detail below as an illustrative example of this category of disease) are two sialic acid-related examples of recycling disorders connected with glycosidase defects. Upon release of free monosaccharides in lysosomal vesicles, the recycling process continues by transmembrane transfer of the sugar into the cytoplasm by an H+/anionic sugar symporter; defects in these transport proteins have been implicated in diseases that include infantile sialic acid storage disease (ISSD) and Salla disease (an adult-onset form of ISSD). These disorders are characterized by the lysosomal accumulation of sialic acid (Verheijen *et al.*, 1999); changes to glycan structure have yet to be described in detail.

Sialidosis

Sialidosis, also called cherry-red spot monoclonus syndrome and mucolipidosis I, is an autosomal recessive lysosomal storage disease caused by a deficiency in lysosomal neuraminidase activity. Neuraminidases (also known as sialidases) comprise a superfamily of hydrolytic enzymes that perform a vital role in the processing and catabolism of sialylated glycoconjugates, which in turn modulates cell proliferation and adhesion, modification of receptors, and clearance of plasma proteins (Reuter and Gabius, 1996). Sialidosis is marked by an abnormal tissue accumulation and urinary excretion of sialylated oligosaccharides and glycoproteins. Type I, or non-dysmorphic, sialidosis is a late onset, mild form of disease, characterized by bilateral macular cherry-red spots, progressive impairment of vision, and myoclonus syndrome (Uhl *et al.*, 2002). Type II, or dysmorphic, infantile-onset form, is characterized by abnormal somatic features, skeletal dysplasia, hepatosplenomagly, and moderate to severe mental retardation (Thomas and Beaudet, 1995). The age of onset and severity of clinical symptoms correlate with the amount of residual neuraminidase activity, suggesting that there might be considerable genetic heterogeneity between patients.

In general, neuraminidase deficiency is due to lesions in the neu1 gene that map to the MHC III cluster on chromosome 6p21.3 (Milner *et al.*, 1997). A variety of mutations have been characterized in the *neu1* gene sequence that range from simple point mutations impacting only the surface residues of the enzyme without directly affecting the deduced active site residues or the core protein structure (Lukong *et al.*, 2000), to intronic mutations, where sequences up to 11 kb in length have been shown to be deleted from the *neu1* gene sequence (Uhl *et al.*, 2002). Mutant neuraminidase enzymes can be classified into three categories on the basis of their biochemical properties. The three groups include mutant enzymes that are catalytically inactive and do not localize to lysosomes, mutants that reach the lysosomes but are catalytically inactive, and variants that are localized to the lysosomes and exhibit residual activity (Bonten *et al.*, 2000). The severity of the clinical symptoms depends strongly on the type of mutation resulting in sialidosis. Patients with Type I sialidosis generally only have mutations from the third enzyme group, while those with Type II sialidosis have one or more mutations from the first two groups (Bonten *et al.*, 2000).

MOLECULAR DETECTION, CLINICAL DIAGNOSIS, AND FUNCTIONAL CHARACTERIZATION OF GLYCOSYLATION DEFECTS

Methods to detect and identify glycosylation defects

Once an alert physician suspects that a patient is suffering from a glycosylation disorder, a molecular defect must be identified at the genetic or protein level to confirm the diagnosis. The most common methods used to detect and characterize molecular changes associated with CDGs are isoelectric focusing analysis (IEF) and gel electrophoresis of serum transferrin (Tf). Transferrin is a commonly misglycosylated protein in CDGs and, because it is secreted into the blood or urine (*Figure 10.1*, Step 4b), it provides an easily obtained, sensitive marker that only yields false positives in patients with alcoholism, uncontrolled fructosaemia, and liver disease (Stibler and Hultcrantz, 1987; Stibler *et al.*, 1988; Jaeken *et al.*, 1996a). In the absence of a glycosylation defect, Tf displays two *N*-linked disialylated bi-antennary chains. In general, these chains are entirely lacking in Tf obtained from CDG Type I patients, whereas Type II patients will display a series of abnormal Tf bands in IEF, indicating the presence of truncated oligosaccharides (Wada *et al.*, 1992; Yamashita *et al.*, 1993; Lacey *et al.*, 2001).

Despite its widespread use, the Tf IEF test is limited by the fact that abnormal Tf readings do not indicate the exact molecular defect responsible for causing a CDG (Harrison *et al.*, 1992; Stibler *et al.*, 1998), requiring additional analysis to pinpoint the exact disease type. Other disorders, including CDG IIb, CDG IIc and, in some cases, CDG Ia, cannot be detected by IEF analysis because they lack Tf abnormalities. These limitations, plus additional challenges inherent in the analysis of non-CDG defects by Tf characterization, have led to the adoption of various complementary methods that include lectin affinity chromatography of glycoprotein hormones (Ferrari *et al.*, 2001), electrospray ionization mass spectrometry (ESI-MS) (Lacey *et al.*, 2001), and testing of cerebral spinal fluid levels (Grunewald *et al.*, 1999). As increasingly sophisticated analytical methods continue to be developed (Mechref and Novotny, 2002; Wormald *et al.*, 2002), characterization of widespread glycosylation changes, not just those specific to Tf, will greatly facilitate the description of congenital glycosylation disorders in the future.

Clinical diagnosis and treatment of CDGs

At present, the clinical diagnosis of glycosylation defects remains technically challenging, often requiring sophisticated biochemical and analytical methods beyond the reach of the clinician. In the future, as a exhaustive compilation of genetic defects become known, one approach for rapid diagnosis is likely to utilize 'gene chips' capable of detecting the various genetic polymorphisms associated with glycosylation-associated diseases. Mirroring the current lack of clinically friendly diagnostic options, few treatment alternatives exist. As has been mentioned on a case-by-case basis for CDGs, monosaccharide supplementation occasionally provides limited therapeutic relief. In the future, if gene therapy becomes a viable technology, replacement of the defective genes may provide another opportunity for treating CDGs. To facilitate both diagnosis and treatment, a thorough molecular and cellular

understanding of the contribution of each defect to the development of the disease is required; methods to achieve this goal are discussed next.

Unravelling the functional contributions of glycosylation defects

Clinical diagnosis of a CDG requires the *identification* of a genetic defect; rigorous proof that a particular defect is actively involved in disease etiology (and is not just incidentally present in the patient) also requires *functional* evidence that the putative molecular abnormality can account for the observed disease symptoms. A battery of techniques has been employed to establish connections between genetic defects and clinically manifest disease; these methods include *in vitro* assays, cell-based methods, and animal models. These efforts often begin with established biochemical and molecular biological methods that include the cloning and overexpression of the mutant form of an enzyme, followed by a detailed characterization of its altered kinetic or substrate-utilization properties. An outstanding example of this approach is the analysis of the sialuria form of UDP-GlcNAc 2-epimerase, where catalytic dysfunction has been tied to specific amino acid mutations (Seppala *et al.*, 1999; Yarema *et al.*, 2001). The next step is to determine what cell-wide metabolic and physiological changes are triggered by the specified metabolic abnormality. Because the process of *N*-linked glycosylation is similar in all eukaryotes, introduction of specific genetic defects into easy-to-manipulate organisms, such as yeast, has been a productive approach for the characterization of formally categorized 'CDG' diseases (Aebi and Hennet, 2001). In addition, a panel of CHO mutants with '*lec*' mutations has provided great insight into both the contribution of individual mutations to disease, as well as uncovering fundamental insights into glycosylation processes (Stanley *et al.*, 1996). Cell-based efforts continue to provide increasingly sophisticated and novel methods to uncover mutations in human cells (Yarema and Bertozzi, 2001). Finally, a powerful, whole-organism approach has been employed, where specific defects are introduced into murine embryonic stem cells and whole-organism effects are ascertained by phenotypic observation of the resultant mouse (Dennis *et al.*, 1999).

Glycosylation changes associated with cancer

Many in-born errors of metabolism, such as the CDGs discussed above, can be traced to an exact mutation in a particular gene. Other illnesses that afflict the human race, by contrast, cannot be ascribed to a single gene (or environmental factor). Instead, they arise from the combined action of many genes, environmental factors, and risk-conferring behaviours (Kikerstis and Roberts, 2002). The multifactorial nature of these diseases, some of which include heart disease, cancer, diabetes (Marx, 2002), and immune disorders (Marshall, 2002), often renders them refractory to effective therapeutic intervention. Accumulating evidence suggests that detection and analysis of glycosylation changes can aid in the understanding, diagnosis, and treatment of these diseases, and therefore constitute a welcome supplement to current methods. Of the many diseases now associated with altered glycosylation, such changes have so far been best characterized for cancer. Cancer, therefore, will be discussed to exemplify the broad range of structural abnormalities that impact oligosaccharides in

the disease state, and how these markers can be used clinically for diagnosis, and increasingly, for therapeutic intervention.

ALTERED GLYCOSYLATION TYPIFIES CANCER CELLS

Abnormal glycosylation is a universal hallmark of cancer cells (*Figure 10.3*). Several factors contribute to the altered patterns of glycosylation found on cancers: 1) certain oligosaccharide structures that appear on normal cells are upregulated; 2) other structures, not typically appearing on normal cells, are newly expressed; and 3) the expression of other epitopes are downregulated or eliminated, exposing previously masked underlying antigens. In general, changes in glycoproteins are associated with glycopeptide enlargement, increased glycopeptide branching at the mannosyl core of *N*-asparagine-linked oligosaccharide, and a larger antennary structure (Ørntoft and Vestergaard, 1999). Altered protein glycosylation is associated with increased levels of *O*-glycosylated mucins, as detected by tumour-specific monoclonal antibodies and variations in sialylation. Glycolipids undergo two major types of glycosylation change: either the incomplete synthesis of normally expressed carbohydrate chains can occur, or carbohydrates not typically expressed in normal cells can be newly synthesized. In the following sections, we describe briefly some of the more common glycosylation changes that accompany cancer; the interested reader is urged to consult the references provided for a more comprehensive description of tumour-associated carbohydrate antigens.

Increased sialylation

One of the most common glycosylation changes in cancer is the increased expression of terminally sialylated sequences in both *N*- and *O*-linked glycans (Pearlstein *et al.*, 1980; Yogeeswaran and Tao, 1980; Yogeeswaran and Salk, 1981). The addition of the negatively charged sialic acid to the otherwise neutral oligosaccharide is important in intercellular interactions (Bhavanandan and Furukawa, 1995), and is a key determinant of cancer grade, invasion, and metastasis (Kim and Varki, 1997). Tumour-associated changes in sialylation are generally attributed to abnormal activity of key sialyltransferases, such as ±-2,6-sialyltransferases (Akamatsu *et al.*, 1996); abnormal activity of these enzymes has been noted in breast cancer (Brockhausen *et al.*, 1995), colon cancer (Yang *et al.*, 1994), and leukaemia (Baker *et al.*, 1987). Finally, precursor accumulation and incomplete synthesis of carbohydrate chains, possibly related to reduced GalNAc content (Brockhausen *et al.*, 1995), allow certain neoplastic cells to display more multi-antennary and hypersialylated *N*-glycans than normal cells (Passantini and Hart, 1988).

Polysialic acid

In most sialoglycoconjugates, sialic acid residues occupy terminal ±-2,6- or ±-2,3-linkages to galactose; one exception is polysialic acid, a linear homopolymer of up to 50 ±-2,8-linked residues (Troy, 1995). Another unique feature of polysialic acid is that, unlike ±-2,6- or ±-2,3-linked sialic acid residues that are widely distributed among all cellular glycans, it is found almost exclusively on oligosaccharides

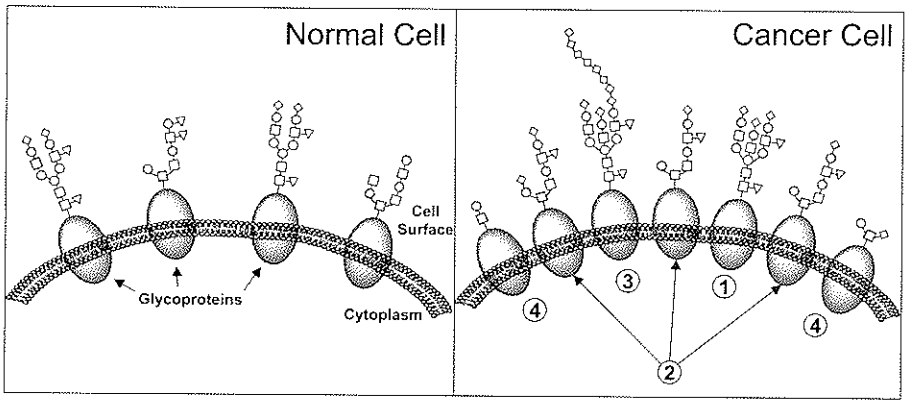

Figure 10.3. Representative differences in cell surface glycosylation between normal and cancer cells. Patterns of glycans presentation of cancer cells (right) typically vary significantly from non-transformed normal cells (left). Changes common to a wide range of malignant cells include: 1) increased branching and sialylation of *N*-linked glycans; 2) increased sialylation of *O*-linked glycans (e.g. to form sialyl-Lewis^x antigens); 3) presentation of masking oligosaccharides, such as polysialic acid; and 4) truncation of certain *O*-linked glycans. Symbols used to represent monosaccharides are defined in *Figure 10.2*.

attached to the neural cell adhesion molecule (NCAM) glycoprotein (Muhlenhoff *et al.*, 2001). Polysialic acid is abundant in the central nervous system during fetal development, but is restricted to regions of the brain associated with synaptic plasticity in the adult (Nakayama *et al.*, 1998). Polysialic acid is aberrantly re-expressed in several cancers, which include neuroblastomas, small cell lung carcinomas, and Wilm's tumour, and is associated with a highly aggressive phenotype and increased metastatic potential (Fukuda, 1996).

Lewis antigen expression

Lewis antigens are relatively unusual terminal oligosaccharide sequences that occur in novel and abundant forms in epithelial cancers. Typical changes are an increase in Lewis^x and Lewis^a antigen presentation, and a loss of Lewis^y structures (Kim and Varki, 1997). For example, Lewis^x antigens are expressed in pancreatic cancers, which normally exhibit Lewis^y antigens (Kim *et al.*, 1988). Malignant colonic mucosa expresses Lewis^x, Lewis^y, and Lewis^b, while normal colon cells only express Lewis^a (Hennet *et al.*, 1995). Increases in Lewis^x and Lewis^a levels have been correlated to poor prognosis in many cancers (Itzkowitz *et al.*, 1990; David *et al.*, 1992; Kakeji *et al.*, 1995). These antigens are typically synthesized by fucosyltransferases FucT I–VIII linking fucose to galactose and GlcNAc residues in both *N*-glycans and *O*-glycans; however, upregulation of these enzymes is primarily noted in colorectal cancers (Ørntoft *et al.*, 1990). A possible molecular explanation for increased Lewis antigen expression in epithelial cancers is the downregulation of the AB blood group transferase (Ørntoft and Wolf, 1988; Ørntoft, 1990). Downregulation of this enzyme has also been shown to be related to cell proliferation (Ørntoft *et al.*, 1996).

Lewis structures frequently appear in sialylated form in transformed cells, and are

associated with an increased risk of metastasis. Specifically, elevated levels of sialyl-Lewis^a (Takada *et al.*, 1991) and sialyl-Lewis^x are implicated in haematogeneous metastasis (Rice and Bevilaqua, 1989; Hession *et al.*, 1990). Sialyl-Lewis^a has also been associated with vascular cell invasion (Rice and Bevilaqua, 1989; Hession *et al.*, 1990), and is upregulated during malignant transformation of pancreatic and gastrointestinal cells (Itai *et al.*, 1988). Sialyl-Lewis^x is frequently expressed in lung, ovary, and gastrointestinal carcinoma cells (Kannagi *et al.*, 1986; Kukowska-Latallo *et al.*, 1990). In the case of gastrointestinal carcinoma, it mediates binding to endothelial cells through E-selectin, thereby contributing to the processes of invasion and metastasis that are associated with poor survival in colon cancers (Sawada *et al.*, 1994).

N-*glycans: multi-antennary terminal formations*

Malignant cells commonly exhibit increased branching of terminal carbohydrates, resulting in multi-antennary *N*-linked oligosaccharides. This aberration is detectable through molecular weight differences, as well as charge and sialic acid content. Increased β-1,6-branching is mediated by *N*-acetylglucosaminyltransferase V (GlcNAc-TV) upregulation, which in turn has been linked to a cellular proliferation signal pathway that modulates the Ets family of transcriptional activators (Ko, 1999). Immunohistochemical studies with the lectin L-PHA binding to segments of tri- and tetra-antennary *N*-glycans have shown increased β-1,6-branching in breast cancers, colon cancers, and melanomas (Cummings and Kornfeld, 1982; Fernandes *et al.*, 1991).

Increased glycan size through branching presents more terminal structures, such as sialyl-Lewis^x, to the surroundings. Tumour progression (Dennis *et al.*, 1987) and increased metastatic potential (Laidler and Litynska, 1997; Seberger and Chaney, 1999) of cancer cells have been attributed to increased *N*-glycan branching. Transfection of human hepatocarcinoma cell lines with over- and underexpressed GlcNAc-TV showed suppressed levels of cell attachment to fibronectin and laminin, and elevated levels of cell migration and invasion (Guo *et al.*, 2001). In another study, the immortalized lung epithelial cell line Mv1Lu was transfected with GlcNAc-TV, resulting in reduced cellular adhesion to fibronectin and collagen. Subsequent transfectant injection into nude mice resulted in a 50% incidence of benign tumours (Demetriou *et al.*, 1995), in agreement with previous reports that GlcNAc-TV induced tumourigenic behaviour in nontumourigenic cells (Cheresh *et al.*, 1984). It has also been suggested that GlcNAc-TV is the primary enzyme responsible for altered glycosylation in malignant cells (Dennis *et al.*, 1999).

Reduction of core 2 structure

Unlike *N*-glycoprotein biosynthesis, where a large oligosaccharide is attached to a nascent peptide, *O*-linked glycoprotein biosynthesis begins by addition of simple 'core' structures to a protein. At least eight different core glycoprotein structures are known to exist, and core structures 1–6 have been detected in human cells. In malignant cells, increased numbers of prematurely truncated *O*-glycan chains of mucin-type glycoproteins can occur, resulting in the reduction of core 2 (Gal-β1,3-

[GlcNAc-β1,6]-GalNAc) structures, and subsequent exposure of previously masked antigens on the cell surface (Dwek *et al.*, 2001). Specific epitopes exposed by reduction of core 2 include the T antigen (the T antigen is GalNAc *O*-glycosidically linked to Ser or Thr residues; a structure common to all *O*-glycans (Brockhausen, 1999)), sialyl-Tn antigen, Tn antigen, and colon-specific Tk antigen (Meichenin *et al.*, 2000). Truncation of *O*-glycan core structures is thought to often result from downregulation of core 2 β-1,6-GlcNAc transferase (Brockhausen *et al.*, 1995), β-1,6-galactosyltransferase, and possibly ±-2,3-sialyltransferase (Ørntoft and Vestergaard, 1999).

Several *O*-glycan related antigens are detected in carcinoma cells, but are rarely, if ever, found in normal cells, making them attractive disease markers. The sialyl-Tn antigen is associated with poor prognosis in colonic, gastric, and ovarian cancers (Itzkowitz *et al.*, 1990; Kobayashi *et al.*, 1992; Takahashi *et al.*, 1993). Tn and T antigens can be recognized by macrophage C-type lectins (Suzuki *et al.*, 1996), sialoadhesion, galectin, and CD33. The T antigen is also detected by galactose receptors and galectins (Suzuki *et al.*, 1996). Useful markers include increased T and Tn expression in advanced stage gastrointestinal cancer (Itzkowitz *et al.*, 1990; David *et al.*, 1992), and sialyl-Tn and Tn in ovarian cancer, where it is associated with poor prognosis (Kobayashi *et al.*, 1992).

O-*glycans: mucins*

Most *O*-glycosylated proteins are mucins, molecules characterized by a high molecular weight and heavily glycosylated Ser/Thr/Pro-rich tandem repeats. Expression of cell surface mucins is often altered in cancer, influencing the cell's interaction with adhesion molecules (McDermott *et al.*, 2001) in a manner believed to promote tumour cell displacement at the onset of metastasis (Hilkens *et al.*, 1992). Mucins can also play a role in suppressing immune response (Denda-Nagai and Irimura, 2000) by masking underlying antigens. Mucins secreted by tumours into urine and the bloodstream have been theorized to act as ligands for haematogenous metastasis. These mucins may either inhibit tumour cell binding, or form aggregate molecules of mucins, leukocytes, tumour cells, and platelets in the bloodstream (Ørntoft and Vestergaard, 1999).

Other tumour-associated glycosylation changes

Some of the more prominent examples of glycosylation changes associated with cancer are outlined above; it should be noted that this list is far from comprehensive, as many more changes have been known for well over a decade (Sell, 1990). A few of these include an abnormal increase in ABO and AB blood group expression (Brockhausen, 1999), a loss of *O*-glycan sulphation, as detected in colon cancer (Yamori *et al.*, 1987), a loss of Sia *O*-acetylation (Kim and Varki, 1997), and CD44 receptor aberrations (Naot *et al.*, 1997). As will be discussed below, in the future, the significance of any particular change, studied in isolation, is likely to diminish in importance and be superseded by the analysis of overall patterns of glycosylation changes that accompany malignancies.

DETECTION AND CLINICAL DIAGNOSIS OF GLYCOSYLATION CHANGES

Immunodetection and lectin binding assays

Over the past three decades, antibodies and lectins have been workhorse reagents in the determination of cancer-related changes in glycosylation. In particular, monoclonal antibodies (MAbs) have facilitated the identification of numerous tumour-associated carbohydrate antigens (Sell, 1990). These versatile reagents are used to recognize surface epitopes newly expressed on cancer cells, and can also be used to detect epitopes that are typically masked on normal cell surfaces but become exposed as a result of oligosaccharide truncation in malignant cells. In other cases, tumour-associated glycans obscure underlying epitopes themselves; an example of a masking epitope of this type is polysialic acid that, incidentally, can itself be detected by monoclonal antibodies (Hayrinen *et al.*, 1995).

Lectins, by definition carbohydrate-binding proteins, also can be exploited to selectively recognize and bind to the aberrant glycan products that accompany disease. Two main classes of lectins are relevant to cancer research. The first category includes endogenous molecules that interact with aberrantly glycosylated cells and play an active role in pathogenesis. The best characterized endogenous lectins are the L-, E-, and P-selectins; in addition to selectins, H-protein, the family of I-type lectins, CD22, CD33, sialoadhesion, myelin-associated glycoprotein, and the Schwann cell myelin protein interact specifically with sialic acids (Varki, 1997). Together, these endogenous lectins and tumour-associated carbohydrate antigens dramatically influence metastatic behaviour, amongst other attributes, of transformed cells. Another category of lectins that also have binding specificities suitable for recognizing carbohydrate antigens overexpressed on human cancer cells, and have become valuable as diagnostic agents, is that derived from exogenous sources, such as plants and crustaceans (Brinck *et al.*, 1998).

Tumour markers used for clinical diagnosis

To facilitate clinical diagnosis, many lectins and MAbs have been evaluated in attempts to find a single marker that indicates that a patient has a particular malignancy. For example, the lectins L-PHA and HPA have proven useful as cancer markers. Increased L-PHA activity is noted in breast carcinoma, atypical hyperplasia, esophageal carcinoma; L-PHA staining is particularly useful as a prognostic indicator of tumour recurrence, patient survival, and the presence of lymph node metastases in colorectal cancer (Seelentag *et al.*, 1998). HPA staining has been proven to be a particularly good indicator of tumour metastatic potential, and poor prognosis in breast (Fenlon *et al.*, 1987; Leathem and Brooks, 1987), colon (Schumacher *et al.*, 1994), stomach (Kakeji *et al.*, 1991), and esophageal (Yoshida *et al.*, 1993) cancers. In general, lectins have value in categorizing broad changes in glycan expression that occur in several types of cancer; by contrast, MAbs offer the potential for detecting unique changes associated with specific types of cancers (Sell, 1990). One commonly used MAb is SM3, which detects 90% of breast carcinoma cells that experience core 2 glycan loss (Burchell *et al.*, 2001), while experiencing negligible activity with MUC1 in normal, pregnant, or lactating breast tissue (Burchell *et al.*, 1987; Girling *et al.*, 1989).

Another example of a diagnostic tumour marker is CA 19-9, which is used for pancreatic and colorectal cancer diagnosis and follow-up, and has been investigated for the detection of bladder cancer (Casetta *et al.*, 1993).

Although they are often sensitive and specific markers for cancer, MAbs suffer from certain limitations that reduce their clinical effectiveness. For example, the efficacy of CA 19-9 in diagnosis of pancreatic cancer is reduced due to crossreactivity with glycan changes that occur in other nonmalignant diseases, as well as variations that occur due to the patient's Lewis and secretor genotypes (Narimatsu *et al.*, 1998). Because of complicating factors of this type, even outstanding examples of tumour markers, such as the prostate-specific antigen (Small and Roach, 2002), are not unambiguously accepted as precise diagnostic and prognostic indicators. Observations that markers such as CA 50 and CA 242, when used in conjunction with each other, greatly increase detection sensitivity of digestive cancers (Carpelan-Holmstrom *et al.*, 1995), have led to the growing realization that trends and patterns of glycan expression must be considered, instead of relying on single markers. Ideally, *every* glycan present on a cancer cell could be analyzed to provide a unique 'glycomic fingerprint' or 'bar code' (Hirabayashi and Kasai, 2002); based on genomic precedent (Takahashi *et al.*, 2001; van't Veer *et al.*, 2002), such a fingerprint would have considerable diagnostic and prognostic value.

Clinical diagnosis: towards glycosylation 'fingerprints'

Obtaining a glycomic fingerprint is a challenging endeavour that requires the determination of overall differences in glycan expression that occur between malignant and normal cells. Innovative methods that include sophisticated variations of chromatographic, electrophoretic, and spectrographic methods (Hirabayashi and Kasai, 2002) are being developed to describe the entire complement of glycans expressed on the cell surface, and catalogue their enormous number and heterogeneity. A first step towards obtaining a glycomic fingerprint is to categorize changes in the expression of glycosylation-related genes by using DNA microarray technology; such analysis is already under way to determine other properties of cancer cells (van't Veer and de Jonge, 2002). However, it is now clear that there can be a significant disconnection between gene expression and the actual 'proteome' in certain cancers (Nelson *et al.*, 2000). Even further variability results from the subsequent modification of the proteins required to produce the glycans in question. Thus, to obtain an *actual* glycomic fingerprint will require the ingenious use of multiple separation and detection technologies (Kuster *et al.*, 2001; Hirabayashi and Kasai, 2002); these techniques, upon fruition, will provide a powerful arsenal of new diagnostic tools and therapeutic approaches (Dwek *et al.*, 2001).

EXPLOITING CARBOHYDRATE CHANGES FOR TREATMENT OF CANCER

Immunotherapy

For almost as long as monoclonal antibodies have been used to detect glycosylation (and other surface) abnormalities associated with cancer, complementary attempts have been made to target cancer cells for selective eradication through the use of

immunotoxins. Immunotoxins are MAb–toxin conjugates designed to bind to tumour-associated antigens newly exposed, or aberrantly overexpressed, on malignant cells (Sell, 1990). As discussed above, unmasking of novel epitopes presents newly exposed antigens suitable for selective targeting by an immunotherapy approach (MacLean *et al.*, 1994; Willett, 2002). Elevated levels of mucins found in pancreatic (Metzger *et al.*, 1984), ovarian and endometrial (Ioannides *et al.*, 1993), breast (Walsh *et al.*, 1993), colorectal (Aksoy *et al.*, 2000), and gastrointestinal (Ørntoft *et al.*, 1990) cancers also provide unique targets for immunotoxins.

An interesting variation to the immunotargeting of aberrantly expressed, but nevertheless natural, antigens is to engineer novel abiotic epitopes onto the cell surface by using emerging cell surface engineering methods. These methods are based on the ability of human cells to incorporate non-natural ManNAc analogues into the corresponding cell surface displayed sialic acids (Keppler *et al.*, 2001). Because sialic acid is often overexpressed in cancer, it is hoped that the novel epitopes will be preferentially displayed on the disease cells. Once on the cell surface, non-natural sialic acid epitopes can function as antigens with therapeutic potential; for example, the non-natural sialic acid 'SiaProp' overexpressed on cancer cells leads to their preferential eradication, compared to normal cells in a mouse model (Liu *et al.*, 2000). Alternately, a newly installed epitope, such as 'SiaLev', can function as a 'chemical handle' (Yarema, 2001) for the delivery of a second antigen (such as the a-Gal epitope (Galili and laTemple, 1997)), complementarily designed toxin (such as a ricin conjugate modelled after immunotoxin conjugates (Mahal *et al.*, 1997, 1999)) or diagnostic agent (Lemieux *et al.*, 1999).

Treatment: cancer vaccines

Currently, cancer therapy concentrates on the destruction and eradication of malignant cells through surgery, radiation, and chemotherapy. In the future, cancer treatments promise to include less invasive methods, such as the use of 'vaccines' designed to stimulate the immune system to prevent the initial occurrence of cancer, and to protect against tumour recurrence and metastasis. Several tumour-associated antigens have been considered for use as cancer vaccines, including melanoma–melanocyte differentiation antigens (e.g. MART-1/Melan-A, gp100, TRP-1), cancer-testes antigens (e.g. MAGE, BAGE, GAGE), mutated antigens (e.g. CDK-4, MUM-1, capase 8), and overexpressed antigens (e.g. MUC-1, α-fetoprotein). Recent approaches to cancer vaccine development have focused on overexpressed cell surface carbohydrate epitopes (Wang *et al.*, 2000). These approaches have proven to be moderately successful, and some antigens have entered clinical trials. Administration of these vaccines has proven to be relatively nontoxic, and has been shown to raise IgM and IgG antibody levels; in some patients, these induced antibodies have also been shown to react with tumour cells (Sabbatini *et al.*, 2000; Wang *et al.*, 2000). In the future, cancer vaccine-based therapies are likely to be used in an adjuvant manner to surgical and chemotherapeutic options.

One factor limiting the adoption of carbohydrates as cancer vaccines is obtaining adequate amounts of highly purified antigen. Purification from natural sources is rendered impractical by the heterogeneity of the complex oligosaccharides found in cells or tissues. Consequently, efforts have turned to the complete chemical synthesis

of these oligosaccharides. Although the synthesis of these complex molecules remains a daunting task, several successes have been reported, including the synthesis of the MBr1 antigen Globo-H (Allen *et al.*, 2000), the adenocarcinoma antigen KH-1 (Lanzavecchia, 1993), the gastrointestinal major and minor N3 antigens (Kim *et al.*, 2001), the lung carcinoma antigen fucosyl GM_1 (Singhal *et al.*, 1991), $Lewis^y$ (Sabbatini *et al.*, 2000), Tn (Hakomori and Zhang, 1997), and Tf (MacLean *et al.*, 1996) antigens. In the future, glycomimetic (discussed below) and other 'chemical glycobiology' approaches (Bertozzi and Kiessling, 2001) may streamline the production of carbohydrate-based cancer vaccines, thus facilitating their widespread use.

Correcting biosynthetic disorders of glycosylation

It has been long recognized that cancer-associated glycosylation changes are useful diagnostic and prognostic tools (Muramatsu, 1993; Nakamori *et al.*, 1993); furthermore, immunotoxins and cancer vaccines represent nascent efforts to exploit carbohydrate changes for therapeutic purposes. Another aspect of carbohydrates that has been alluded to is that, instead of just being a useful correlate of cancer, their modification plays a functional role in the development and progression of the disease. Of particular relevance to cancer, aberrant glycosylation impairs basic cellular functions that include signal transduction, cell–cell and cell–extracellular matrix interactions; cell growth, maturity, differentiation, and possibly apoptosis, are also influenced (Kellokumpu *et al.*, 2002). By altering the natural adhesive (Zhu *et al.*, 1984; Zhu and Laine, 1985) and invasive (Yogeeswaran and Salk, 1981; Collard *et al.*, 1986) properties of a cell, these changes often lead to abnormal cell proliferation, differentiation, and metastasis (Ryder *et al.*, 1992; Rhodes, 1996; Jordinson *et al.*, 1999; Couldrey and Green, 2000).

Because the physical presence of defective glycans plays an active role in the development of the cancer disease phenotype, reversing changes to oligosaccharide structures holds therapeutic value. However, unlike congenital disorders of glycosylation, where the exact genetic defect responsible for the disease is defined, relatively little is understood of the underlying molecular mechanisms responsible for tumour-associated changes in oligosaccharide structure, making it impossible to rationally intervene and correct these defects. In general, altered glycosylation in malignant cells is currently believed to be largely caused by changes in the regulatory mechanisms that control glycosyltransferases (Schachter and Brockhausen, 1992; Brockhausen and Schachter, 1997; Kellokumpu *et al.*, 2002); methods to modulate transferase activity are therefore likely to be applicable to cancer-related (and other) glycosylation aberrations, and will be discussed later.

Additional diseases characterized by altered glycosylation

Glycosylation markers associated with disease have been described most thoroughly at a genetic and structural level for congenital disorders and cancer, respectively. Important glycosylation changes also accompany a wide range of other diseases, whose scope and variety is widening by the day. We now briefly present diabetes, Alzheimer's disease, IgA nephropathy, and infectious agents as a sampling of the

diseases in which the role of carbohydrates play a role, to show the diversity of responses incurred by these molecules.

DIABETES AND ATHEROSCLEROSIS

Diabetes has been closely linked to the accumulation of advanced glycation endproducts (AGEs). AGEs are created nonenzymatically via the glycosylation of proteins and lipids in a process that begins with the formation of reversible bonds between glucose and amino proteins; subsequent rearrangement and polymerization forms irreversibly bound AGEs (Vlassara *et al.*, 1994; Cooksey *et al.*, 1999). AGEs are characterized by structural and physicochemical diversity, such as fluorescence and reticulated structures (Turk *et al.*, 2001). AGE formation can result from exogenous factors, such as exposure to smoking or a fatty, carbohydrate-rich diet (Cooksey *et al.*, 1999); accumulation of AGEs accelerates with ageing, and accompanies certain diseases, including diabetes.

In an animal model, diabetes occurs a few weeks *after* AGE levels increase, suggesting that AGE accumulation may play a causative role in disease progression (Tan *et al.*, 2002). Interestingly, drugs that inhibit AGE formation ameliorate tissue damage that occurs in diabetes (Vasan *et al.*, 2001). The exact role that AGEs play in the development of diabetes is not well defined, but, in general, aberrant and increased AGE levels have been directly implicated in several pathological events (Brownlee *et al.*, 1986; Darby *et al.*, 1997), including abnormal smooth muscle cell proliferation in arterial walls (Akimoto *et al.*, 2001), increased vascular permeability, increased strength of cell adhesion, and induced migration of macrophages and T cells into the intima (Wautier *et al.*, 1996). AGE formation also deleteriously interferes with non-receptor-mediated events of molecular conformation, enzymatic activity, low-density lipoprotein (LDL) physiology, degradative capacity (Steinbrecher and Witztum, 1984; Tan *et al.*, 2002), extracellular matrix component synthesis (Brownlee *et al.*, 1988), and cross-linking induction (Odani *et al.*, 2001). Finally, receptor-mediated events, such as inflammation promotion (Brownlee *et al.*, 1986; Vlassara *et al.*, 1992), induction of oxidative stress, and endothelial dysfunction (Tan *et al.*, 2002), are also attributed to aberrant glycosylation.

Regardless of the exact role of AGEs in diabetes, they serve as useful markers for this disease, as well as for diabetes-related atherosclerosis. As a corollary to AGE formation, diabetes is characterized by prolonged exposure to hyperglycaemia, which leads to the increased risk of additional disorders, most notably heart disease; approximately 80% of diabetic deaths are attributed to atherosclerosis (Seberger and Chaney, 1999). In addition, aberrant *O*-GlcNAc post-translational modification of proteins occurs as a result of high glucose content (Akimoto *et al.*, 2001) that, in turn, results from increased insulin resistance (Cooksey *et al.*, 1999; Vosseller *et al.*, 2002). Other glycosylation changes in diabetes include variations in fucosylation and sialylation patterns (Cohen-Forterre *et al.*, 1990; Wiese *et al.*, 1997).

ALZHEIMER'S DISEASE

As the populations of developed countries age, Alzheimer's disease is rapidly becoming a dreaded ailment on a personal level, and a formidable burden to society

as a whole. Although it is known that Alzheimer's disease is caused by abnormal processing of the amyloid precursor protein (APP), many of the molecular details of disease progression remain mysterious, and no effective treatments have emerged so far. Recent reports have linked several changes in glycosylation with APP processing defects, and changes to brain histology further indicate that glycans are intimately involved in pathogenesis. Glycosylation changes are associated with several molecular players in the development of Alzheimer's disease, including APP itself. This protein is typically glycosylated, and changes in glycosylation interfere with its intracellular sorting (Tagawa *et al.*, 1992; Yankner, 1996). Additional glycosylation changes can occur to the enzymes that process APP. For example, the β-secretase protein Asp-2 has four potential sites for *N*-glycosylation, and variability in glycosylation affects the activity of this protease towards APP (Charlwood *et al.*, 2001).

Besides the glycosylation changes to APP and proteases that process this protein, there is a significant increase in the overall production of *O*-glycosylated proteins in neuritic plaques and neurofibrillary tangles found in the brains of Alzheimer's disease patients (Guevara *et al.*, 1998). Similar widespread changes in *O*-glycosylation have been linked to pathogenic changes in neuronal plasticity (Espinosa *et al.*, 2001). Because these specific glycosylation patterns are closely correlated with the hallmark lesions found in Alzheimer's patients (Guevara *et al.*, 1998), it is not surprising that glycosylation patterns are emerging as a sensitive and specific diagnosis tool in the detection of this disease. In particular, analysis of the glycosylation patterns of acetylcholinesterase and butyrylcholinesterase from cerebrospinal fluid provides a sensitive and specific diagnostic tool that has greater than 90% accuracy (Saez-Valero *et al.*, 1997, 2000).

IgA NEPHROPATHY

Immunoglobulin A nephropathy (IgAN) is a common form of glomerulonephritis. Although the exact pathogenesis of this form of kidney disease remains undefined, considerable evidence implicates the involvement of abnormally glycosylated IgA. Human IgA is highly and, for a secreted molecule, unusually, glycosylated; specifically, the IgA1 subclass contains five *O*-glycan sites in the CH1 and CH2 hinge domain, and nine *O*-glycan sites in the proline-rich Fab and Fc hinge domain of its heavy chain. Potential sites for *O*-glycan attachment are usually occupied by core GalNAc residues that are extended with galactose via a β-1,3 linkage; in turn, the galactose is typically elaborated with sialic acid via an ±-2,3- or ±-2,6-linkage (Kiki *et al.*, 2001). Structural abnormalities found in IgAN have been characterized by lectin binding assays (Allen *et al.*, 2001) and mass spectrometry (Kiki *et al.*, 2001). The binding of GalNAc-specific lectins to IgAN samples has been found to be significantly higher than in normal samples. Similarly, mass spectrometry studies demonstrated that IgAN samples shift to a lower molecular weight range than normal IgA1 samples. Together, these results show that the galactose and sialic acid content of glomerular monomeric IgA and serum IgA from IgAN patients was significantly reduced, compared to normal levels. In contrast to monomeric IgA, polymeric λ-IgA1 is oversialylated with decreased levels of Gal β-1,3-GalNAc and GalNAc (Leung *et al.*, 2002). This oversialylation is thought to increase the overall negative charge, and may contribute to increased levels of anionic ICs in the mesanglial region (Isaac and Miller, 1982).

Abnormal glycosylation and sialylation observed in IgAN patients has been postulated to be caused by altered amino acid sequences in the IgA molecule, changes in glycosidase, galactrosyltransferase, and/or sialyltransferase activity (Leung *et al.*, 2002), or relative levels of Th1 and Th2 cytokines (Chintalacharuvu *et al.*, 2001). Regardless of their exact molecular origin, truncated monomeric *O*-glycan structures are believed to contribute to the overall pathogenic role of IgAN (Kiki *et al.*, 2001) by promoting the self-aggregation of IgA molecules; an abnormally high 70% of glomerular IgA from IgAN patients is polymeric (Montiero *et al.*, 1984). A decrease in sialylation may directly contribute to IgA1 deposition; alternately, the abnormal form of IgA may be recognized as an antigen, and aggregation may occur by the action of normal antibodies (Novak and Julian, 1999). Aberrantly glycosylated, polymeric IgA has been shown to deposit in glomeruli more readily, due to enhanced lectin reactivity (Coppo *et al.*, 1991), increased binding (Emancipator *et al.*, 1993), enhanced mesanglial cell (MC) apoptosis, decreased MC proliferation (Amore *et al.*, 1998), and upregulated integrin activity. Abnormal IgA may also alter normal complement fixation pathways and increase activation of the C3 neoantigen, resulting in enhanced nitric oxide activity in the MC. Together, these abnormalities eventually lead to glomerular inflammation, sclerosis, and renal failure (Chintalacharuvu *et al.*, 2001).

INFECTIOUS DISEASE

The first step in almost all infectious disease is the adhesion of a pathogen to the tissue of the host organism. These interactions are often initiated by the binding of lectins located on the surface of incoming pathogen to oligosaccharides of the requisite structure displayed on the surface of the host cells. An emerging treatment strategy is to provide soluble analogues of cell surface carbohydrate ligands, with the goal of having these soluble mimics occupy the receptors on the pathogen, thereby preventing them from binding to the cell surface and initiating infection (Yarema and Bertozzi, 1998). In most cases, the pathogen also has glycosylation features that play key roles, many of which are just now being identified, in pathogenesis; a step-by-step analysis is required to provide additional therapeutic opportunities in the future.

Pathogenic bacteria exploit host carbohydrates during infection

Many pathogenic bacteria possess surface lectins that bind to complementary oligosaccharide components on the surfaces of the cells of their intended host. Pathogenic bacteria infect a range of organs, including the urinary, neural, intestinal, respiratory, stomach, endothelial, and genital tissues, and exploit both glycolipids and glycoproteins as binding partners (Ofek *et al.*, 1996; Sharon and Ofek, 2000). Specific bacteria that utilize glycolipids as adhesion epitopes include *Escherichia coli* strains P, S and K99, *Haemophilus influenzae*, *Neisseria gonorrhoea*, *N. meningitidis*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *S. suis*. Glycoprotein-binding pathogenic bacteria include *E. coli* Type 1, as well as the CFA/1 and K1 strains, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, and *Salmonella typhimurium*. It might be noted that, in addition to bacteria, many eukaryotic parasites, such as the intestinal pathogen *Entamoeba histolytica* (Yi *et al.*,

1998), bind to specific carbohydrate epitopes as an initial step in colonization of the host organism.

Bacterial carbohydrates are involved in pathogenesis

The outer membrane of most Gram-negative bacteria contains lipopolysaccharides (LPS). LPS are complex molecules consisting of a membrane-anchoring phosphoglycolipid (lipid A) covalently linked to a hydrophilic heteropolysaccharide. The polysaccharide moiety (the O-antigen) consists of a core oligosaccharide attached to a polymer of oligosaccharides. The exact LPS structure is highly variable, differing among bacterial species, and even strains of the same species. LPS contains seven- and eight-carbon monosaccharide residues not usually found in mammalian cells; these unusual sugars are thought to contribute to its high immunogenicity. LPS (originally known as endotoxin) triggers sepsis when shed from the bacterial surface. This condition annually affects up to 300 000 patients in the United States alone, approximately 25% of whom progress to fatal septic shock (Mayeux, 1997; Alexander and Rietschel, 2001). Efforts are under way to find less toxic mimetics of LPS to serve as vaccines to prime the immune system, and thus limit bacterial replication and LPS production during bacterial infection.

Carbohydrate-mediated antigen masking is a hallmark of infectious agents; a widely conserved function of the surface oligosaccharides of pathogens is to shield underlying immunogenic epitopes from host immunodetection. A polysialic capsule plays this role for several species of pathogenic bacteria, including *N. meningitidis* (Kahler *et al.*, 1998). Other bacteria, including different strains of *N. meningitidis*, utilize single sialic acid residues to mask antigenic carbohydrate epitopes (Estabrook *et al.*, 1997). Many viruses, including influenza and HIV, which will be discussed below, also utilize oligosaccharides to shield antigenic amino acid sequences in their coat proteins. Finally, although not infectious agents in the common sense, cancer cells within the human body are essentially foreign bodies displaying potentially immunogenic antigens; oligosaccharides can also protect these cells from immunosurveillance (Burchell *et al.*, 1993). Hypersialylation, in particular, acts as a mask on the cell surface to hide tumour-associated antigenic epitopes from antibody recognition, and to decrease natural killer cell access to the malignant cells (Sotiropoulou *et al.*, 2002).

Influenza

The influenza virus is one of the best characterized human pathogens. It initiates colonization of host cells by the multivalent binding of haemagglutanin (HA) molecules on the viral envelope to sialic acid-terminated oligosaccharides displayed on the host cell. To establish host specificity, this virus can generally distinguish between Sia-α-2,3-Gal and Sia-α-2,6-Gal linkages, and also between the NeuAc and NeuGc forms of sialic acid; for example, influenza infecting human cells over the past 25 years have evolved to target α-2,6-linked NeuAc as the preferred ligand (Suzuki *et al.*, 2000). The viral coat contains two proteins. The first one, HA, binds to sialic acid ligands to initiate infection, as mentioned. The other protein, neuraminidase (NA), acts to cleave sialic acids from surface receptors to facilitate virus release into

the cell after it becomes encapsulated in endocytotic vesicles. NA can also function to release the virus from extracellular inhibitors to promote spread to neighbouring cells (Wagner *et al.*, 2002).

Both of the surface proteins of the influenza virus are glycosylated. An interesting feature of the glycosylation of these proteins is that, because the viral genome is replicated by a polymerase lacking an editing function, mutations in the amino acid sequence occur frequently, and often affect potential sites of *N*-glycosylation. Consequently, viral mutants with variations in both *N*-glycan number and location easily arise (Mir-Shekari *et al.*, 1997). This genome plasticity, in particular with regards to *increased* oligosaccharide presentation, enhances population survival and viral spread by masking peptide antigens normally expressed on these glycoproteins, thereby rendering the immune response of the host less effective. While increased glycosylation of coat proteins is advantageous for certain aspects of viral life cycle, such as evasion of the immune system, it is disadvantageous for other viral functions, including binding of HA to cell surface receptors, a necessary step in the cytopathogenecity and virulence of the virus. Consequently, mutations that impact glycosylation represent a trade-off between loss of function and the ability to survive in a hostile environment. Glycosylation sequons also have specific functions. For example, they influence proteolytic cleavage of HA into HA1 and HA2, thus promoting virus fusion and replication (Huang *et al.*, 1981), both of which are qualities that must be preserved for viral activity. The net result is that variations in glycosylation are subject to conservative selection pressures that limit the actual glycan complement to a subset of all possible structures (Mir-Shekari *et al.*, 1997).

Human immunodeficiency virus (HIV)

Human immunodeficiency virus Type 1 (HIV-1) is a highly complex retrovirus that primarily infects CD4+ T lymphocytes and monocyte-derived macrophages (MDM) through the interaction of envelope glycoprotein gp120 with the CD4+ surface marker. Gp120 also binds to the alternate carbohydrate-containing ligand, glycolipid galactosylceramide (GalCer), thereby enabling HIV-1 to enter CD4-negative neural and epithelial cells (Fantini *et al.*, 1993). Recently, amino acid residues 650–685 of HIV-1 envelope protein gp41 have also been shown to function as a lectin by binding to GalCer (Alfsen and Bomsel, 2002). Soluble analogues of GalCer have been synthesized towards the goal of providing molecular decoys that occupy the ligand binding pockets of these receptors, thereby preventing them from binding to cell surface GalCer (laBell *et al.*, 2002). Efforts are under way to define the exact contribution of the oligosaccharide component of gp41 and gp120, as well as cellular glycans, to viral life cycle in order to identify additional therapeutic targets and treatment strategies for the management of HIV-caused disease.

Collectively, viral carbohydrates are also necessary for HIV-1 entry into host cells. They establish important aspects of the viral phenotype, and influence its interaction with the immune system, and define several specific aspects of viral behaviour. Reminiscent of influenza, HIV-1 coat proteins, gp120 and gp41, are highly glycosylated (carbohydrates comprise up to 50% of the total mass of these envelope glycoproteins (Perrin *et al.*, 1998)). The oligosaccharides act to mask antibody neutralization sites

and reduce host immune response (Schonning *et al.*, 1996; Stamatatos and Cheng-Mayer, 1998). In general, the oligosaccharide components of gp120 play a significant role in determining its overall conformation, and subsequently, its processing and ability to interact with CD4 (Fennie and Lasky, 1989; Pal *et al.*, 1989). A specific function of subunit gp120 is to induce virus fusion by interacting with CD4 and a co-receptor (e.g. CXCR4 and CCR5). This interaction triggers conformational changes in glycoprotein structure that allows the hydrophobic domain of the transmemembrane glycoprotein gp41 to interact with the cell membrane and induce fusion (Ogert *et al.*, 2001). Decreased *N*-glycosylation of the CXCR4 receptor results in increased co-receptor activity, a characteristic that is dominant in early HIV-1 infection and virus transmission (Chabot *et al.*, 2000). Unravelling of the specific contributions of glycans in each step to HIV disease progression is an ongoing process, and is progressing towards the goal of developing novel, and urgently needed, therapeutic and prophylactic agents.

Beyond detection and diagnosis: towards active intervention in disease

CARBOHYDRATES AND GLYCOMIMETICS AS DRUGS

Over the past few years, the study of glycosylation defects in disease has undergone a fundamental transition. Previously, efforts were concentrated towards descriptively cataloguing changes that accompany disease; identification of glycosylation defects served as useful markers for the diagnosis of disease. Today, efforts have been extended to exploit carbohydrates as medicines to actively intervene in a disease. As discussed, oligosaccharides have the potential to serve as vaccines against cancer or septic shock, and function as molecular decoys to deter infectious pathogens (Yarema and Bertozzi, 1998; Bertozzi and Kiessling, 2001). Unfortunately, carbohydrates themselves are notoriously poor therapeutic agents; when introduced into the body, they are rapidly cleared from the bloodstream, limiting their use to short-term applications, such as the prevention of myocardial reperfusion injury following a stroke or heart attack (Buerke *et al.*, 1994). Many attractive applications, such as the long-term management of chronic inflammatory conditions or the prevention of metastatic spread of cancer, remain unavailable because of the prohibitive quantities of difficult-to-obtain oligosaccharides required.

Glycomimetics

The incredible potential that oligosaccharides hold for the treatment of disease, coupled with the practical difficulties that prevent clinical realization of their potential benefits, have spurred research efforts into the development of 'glycomimetics'. The design of these molecules is predicated on the identification and retention of the minimal functional domain that allows a parent oligosaccharide to interact with its cognate binding partner. Conversely, structural elements that are wholly dispensable are replaced with a simplified, non-carbohydrate scaffold. The net effect is to distill crucial components for a particular binding interaction into a non-carbohydrate, more drug-like molecule (Gruner *et al.*, 2002). In the future, such glycomimetics are likely to be broadly employed in the treatment of disease (Yarema and Bertozzi, 1998;

Bertozzi and Kiessling, 2001); sialylmimetics, discussed below, are a notable example of these compounds.

Sialylmimetics

Sialic acids have been implicated in numerous aspects of human disease, such as contributing to the development of the cancer disease phenotype, and serving as ligands in cell–microbe interaction. Consequently, there is wide interest in synthesizing modified and natural forms of sialic acid. However, the complexity of sialic acid chemistry, particularly in the area of functional group manipulations and glycosidation reactions (von Itzstein and Kiefel, 1997), combined with the previously discussed limitations inherent in using sugars therapeutically, has led to interest in developing mimetics of sialic acid that retain the structural features essential for interaction with a particular protein, but have improved pharmacological profiles and are structurally simpler (Bradley *et al.*, 2001).

This interest has resulted in the development of mimetics towards a range of sialic acid-containing structures, the most notable of which are α-2,6-linked thiosialosides (Bradley *et al.*, 2001), and sialyl-Lewis^x. Physiologically stable glycomimetics of sialyl-Lewis^x that are based on aryl-C-glycosides are resistant to enzymatic and acidic digestion (Kuribayashi *et al.*, 1998). These compounds, and ones similar to them, have been shown to exhibit comparable affinity for E-selectin, when compared to sialyl-Lewis^x (Kuribayashi *et al.*, 1998; Bradley *et al.*, 2001). Glycyrrhizin (GM1292), a naturally occurring glycomimetic of sialyl-Lewis^x, and its structural derivative, GM3290, can reduce the degree of neutrophil adhesion in the myocardium of rabbit hearts (Kilgore *et al.*, 1988). By mimicking the sialyl-Lewis^x which is present along the myocardial cell surfaces, GM1292 and GB3290 competitively bind to the leukocyte-displayed selectins, thus lowering the level of immune response in the tissue. Carbohydrate-based mimetics such as these significantly reduce myocardial infarct size after periods of prolonged regional ischemia (Kilgore *et al.*, 1988). The increased pace and participation of researchers in the sialylmimetic field makes it likely that pharmaceutical agents will be marketed within the next decade (Kiefel and von Itzstein, 2002).

'REPAIR' OF GLYCOSYLATION DISORDERS AT A MOLECULAR LEVEL

Small-molecule modulators of biosynthetic enzymes

As discussed above, nascent efforts are under way to exploit oligosaccharides (or their mimetics) as medicines for the treatment of certain diseases. In other cases, delivery of an exogenously supplied compound may be ineffective, and it will be necessary to delve within a cell, repair the metabolic defect, and enable the glycosylation machinery to produce the correct complement of glycans on its own. As mentioned earlier, gene therapy may one day be a viable method to permanently repair metabolic defects, such as those found in CDGs. For now, efforts that are under way to develop drugs that target the enzymes involved in glycosylation attempt to modulate, rather than ablate, the activity of target enzymes. Two examples of small

molecule modulators of enzyme activity include inhibitors of UDP-GlcNAc 2-epimerase (Blume *et al.*, 2002), and polysialic acid biosynthesis (Mahal *et al.*, 2001); compounds like these may be employed for treatment of sialuria and metastatic cancer, respectively. The pathology associated with the expression of GlcNAc-Ts in cancer and diabetes-induced cardiac hypertrophy suggest that inhibitors of these enzymes, and glycosyltransferases in general, also hold therapeutic value (Dennis *et al.*, 1999). In certain circumstances, it may be desirable to stimulate, instead of inhibiting, glycosyltransferases; for example, carcinoma cell *O*-glycan extension and sialylation can reduce metastatic potential (Bresalier *et al.*, 1991). Finally, in addition to cancer, the use of glycomimetics to modulate glycosylation enzymes shows considerable promise in the treatment of viral infections, such as hepatitis B, and glucosphingolipid storage disorders, such as Gaucher's disease (Dwek *et al.*, 2002).

Glycosyltransferase inhibitors

The abnormal glycosylation patterns that are associated with various diseases are most often attributed to defects associated with glycosyltransferases. The rational design of glycosyltransferase inhibitors is a difficult task, due to the fact that they utilize complex four-partner transition states and have weak enzyme-natural substrate binding rates. In addition, there remains a fundamental lack of information about many aspects of the glycosyltransferase catalytic mechanism and 3-D enzyme structure (Tvaroska *et al.*, 2000; Unligil and Rini, 2000). Despite these obstacles, effective inhibitors that are structurally altered analogues of carbohydrates designed to simulate the shape and functionality of the natural or ground state of the substrate are now being reported. The most common structural modification is the replacement of the glycosidic or endocyclic oxygen atom with a heteroatom or carbon atom (Compain and Martin, 2001). So far, iminosugars and carbasugars, proven inhibitors of the mechanistically similar glycosidases (Legler, 1990), are the most promising candidates for glycosyltransferase inhibitors, and have become an important area of research (Tvaroska *et al.*, 2000; Unligil and Rini, 2000).

Iminosugars, the first major category of compounds to be tested as glycosyltransferase inhibitors, become protonated under physiological conditions to form a cation that interacts strongly with an anionic group in the active site of the target enzyme. Pyrrolidinols, one of the main classes of iminosugars, have been modified to produce inhibitive effects on β-1,4-galactosyltransferases (Takayama *et al.*, 1999; Saotome *et al.*, 2000), ±-1,3-fucosyltransferases (Springer and Lasky, 1991; Wang *et al.*, 1993), ceramide glucosyltransferases (Butters *et al.*, 2000; Cox *et al.*, 2000). Similarly, piperidinol-based compounds have been shown to inhibit ±-1,4-galactosyltransferases (Jefferies and Rowen, 1997; Cox *et al.*, 2000), as well as ±-1,3-fucosyltransferases and ceramide glucosyltransferases. Carbasugars, analogues of curanoses or pyranoses in which the ring oxygen is replaced by a methylene group, are also attractive because of their chemical stability and proven use as glycosidase inhibitors (McCasland *et al.*, 1966; Suami and Ogawa, 1990). Carbasugar-based structures have provided a variety of sialyltransferase (Korytnyk *et al.*, 1980), fucosyltransferase (Dean *et al.*, 1992), galactosyltransferase (Yuasa *et al.*, 1995), and glucuronosyltransferase (Noort *et al.*, 1990; Battaglia *et al.*, 1995) inhibitors. Glycosyltransferase inhibitors based on structures such as C-glycosides have also

been reported, although their biological activity has yet to be characterized in detail (Compain and Martin, 2001).

Concluding comments

The many disease-related glycosylation markers now known are the result of a close collaboration between physicians who, upon suspecting that a glycosylation defect may be the cause of a clinical disorder, alert glycobiologists, who then seek to uncover the molecular basis of the disease. This relationship is highly synergistic; as more disorders are described at a molecular level, clinicians become increasingly aware of the potential impact of glycosylation defects on human health, and are more likely to seek the input of glycobiologists. As a result, the pace of discovery has accelerated in recent years, and shows no sign of abating. Another ongoing development is the continued expansion of research horizons in several directions. For example, in the past, the study of congenital disorders has focused on discovering the genetic origin of the disease, while the analysis of cancer-related abnormalities has centred on describing changes to oligosaccharide structure. Now, research on congenital disorders increasingly seeks to describe structural changes to glycan structures, and cancer researchers are urgently searching for the genetic (and proteomic) basis of the structural abnormalities they observe and, in both cases, attempts are being made to link these genetic and structural factors to a functional role in disease development. Efforts are also under way to use carbohydrates therapeutically, for example as vaccines, ligands for immunotoxins, or molecular decoys against pathogens.

It is noteworthy that, even though some aspects of overall carbohydrate metabolism (*Figure 10.1*) have been considered outside the domain of strictly defined 'glycosylation disorders', the interconnected networks that comprise this system share many close metabolic linkages. Defects located anywhere in these networks are likely to have indirect effects felt throughout the system, and have the potential to play important roles in the development of a disease phenotype. In reality, even the consideration of carbohydrate metabolism in its totality is likely to prove too narrow a focus. This point is illustrated by efforts to elucidate the exact contributions of glycosyltransferases to the overall patterns of cellular glycosylation. Specifically, *in vitro* experiments have shown that transferase activity is affected by many factors, including the concentration of metal ions with proteins and lipids, post-translational modifications of enzyme and accessory proteins, and gene expression (Brockhausen and Schachter, 1997). The situation *in vivo* is even more complex; final oligosaccharide structures are thought to be specified by spatial distribution of glycosyltransferases in the ER and Golgi apparatus, sugar nucleotide availability, and inter-enzyme competition for acceptor intermediates. Complementing these empirical observations are theoretical principles emerging from the field of mathematical modelling of metabolic networks that indicate that control distribution among internal pathway enzymes can be small, compared to overall cell properties and factors extrinsic to the pathway itself; glycogenesis in skeletal muscle, for example, is controlled more by external, rather than inside, factors (Lambeth and Kushmerick, 2002). Clearly, unravelling each feature that contributes to the overall patterns of glycan expression associated with disease will not be a trivial process, but with the unfolding genomic, proteomic, and glycomic tools now available, these efforts are becoming increasingly

feasible, and promise to have a profoundly beneficial impact on human health in the near future.

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