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Circulating blood and platelets supply glycosyltransferases that enable extrinsic extracellular glycosylation

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Abstract

Glycosyltransferases, usually residing within the intracellular secretory apparatus, also circulate in the blood. Many of these blood-borne glycosyltransferases are associated with pathological states, including malignancies and inflammatory conditions. Despite the potential for dynamic modifications of glycans on distal cell surfaces and in the extracellular milieu, the glycan-modifying activities present in systemic circulation have not been systematically examined. Here, we describe an evaluation of blood-borne sialyl-, galactosyl- and fucosyltransferase activities that act upon the four common terminal glycan precursor motifs, GlcNAc monomer, Gal(β3)GlcNAc, Gal(β4)GlcNAc and Gal(β3)GalNAc, to produce more complex glycan structures. Data from radioisotope assays and detailed product analysis by sequential tandem mass spectrometry show that blood has the capacity to generate many of the well-recognized and important glycan motifs, including the Lewis, sialyl-Lewis, H- and Sialyl-T antigens. While many of these glycosyltransferases are freely circulating in the plasma, human and mouse platelets are important carriers for others, including ST3Gal-1 and β4GalT. Platelets compartmentalize glycosyltransferases and release them upon activation. Human platelets are also carriers for large amounts of ST6Gal-1 and the α3-sialyl to Gal(β4)GlcNAc sialyltransferases, both of which are conspicuously absent in mouse platelets. This study highlights the capability of circulatory glycosyltransferases, which are dynamically controlled by platelet activation, to remodel cell surface glycans and alter cell behavior.

Key words: extrinsic glycosylation, glycosyltransferases, platelets, serum

Introduction

In the canonical understanding of the glycosylation process, glycosyltransferases residing within the intracellular secretory apparatus assemble glycan structures on nascent proteins and lipids in transit. However, many of these glycan-constructing enzymes are also in systemic circulation. Over a period of decades, the clinical association of some of these blood-borne glycosyltransferase activities with pathologic states, such as liver diseases (Kim et al. 1972), atherosclerosis (Gracheva et al. 1999), diabetes (Reddi et al. 1976) and in malignant states such as leukemia (Kuhns et al. 1980), colon (Weiser et al. 1976), breast (Ip and Dao 1978) and lung cancers (Holmes et al. 1986) has been well-documented. Moreover, there have been sporadic reports of the glycan features on the surfaces of cancer cells being remodeled by these systemic glycosyltransferases (Beek et al. 1975; Hounsell et al. 1997). Nevertheless, the extent and physiologic value of these blood-borne glycan-modifying enzymes have remained enigmatic, due largely to the long-held perception that a source of activated sugar-donor substrates necessary to drive extracellular glycosylation reactions does not exist. This modification of cell surface glycans mediated by extracellular glycosyltransferases will be termed extrinsic glycosylation, or extrinsic glycan remodeling.

Two recent key findings rejuvenated the notion that extracellular glycosyltransferases may have important physiologic roles. First, data show that circulatory sialyltransferase (SiaT) ST6Gal-1, long recognized as an integral component of the acute phase response (Kaplan et al. 1983; Jamieson et al. 1993), is an important systemic regulator of blood cell production in the marrow (Nasirikenari et al. 2014). Animals deficient specifically in the blood-borne pool of ST6Gal-1 exhibited exaggerated responses to T helper cell inflammatory stimuli attributable to overly robust production of granulocytes (Jones et al. 2010) and eosinophils (Nasirikenari et al. 2010), and had attenuated ability to produce the sialyl-epitope on the Fc region of immunoglobulin G (IgG) critical in immunosuppressive IgG activity (van der Harst et al. 2012; Jones et al. 2016). Moreover, hematopoietic stem and progenitor cells in the marrow absolutely require extracellular ST6Gal-1 for α2,6-sialylation on their cell surface, regardless of the intrinsic expression status of the intrinsic ST6Gal-1 (Nasirikenari et al. 2014). Second, circulating platelets have been implicated to be carriers of the activated sugar-donor substrates that can be released upon activation (Wandall et al. 2012). We recently provided proof that activated platelets can supply activated sialic acid-donor substrate in sufficient concentration to efficiently drive extrinsic sialylation by ST6Gal-1 (Lee et al. 2014).

ST6Gal-1 generates the α6-sialyl linkage to galactose (Gal) moiety on the Type-II lactosamine (LacNAc) disaccharide, which is comprised of a Gal linked to an *N*-acetylglucosamine (GlcNAc) through a β4 linkage, also abbreviated as Gal(β4)GlcNAc. ST6Gal-1 generated α6-sialyl linkages have been implicated in modulating integrin-mediated adhesive pathways important not only in trafficking of inflammatory cells in the periphery, but also localization of hematopoietic stem cells within the specific micro-environmental niches with the bone marrow (Teixidó et al. 1992; Semel et al. 2002). Type-II LacNAc, together with Type-I LacNAc (Gal(β3)GlcNAc) and the mucin-type Core 1 (Gal(β3)*N*-acetylgalactosamine (GalNAc)) are the three most common terminal glycan motifs on cell surface and secreted glycoproteins. Most of the glycan structures with well-established biologic relevance are glycosylated extensions of these motifs. These include the sialyl Lewis epitopes that serve as ligands for selectin mediated homing of leukocyte and stem cells (Hallmann et al. 1991; Foxall et al. 1992), and the cancer-associated sialyl-T and T antigens (Cazet et al. 2010; Ju et al. 2011).

In this report, we show that the sialyl-, galactosyl- and fucosyltransferase (FucT) activities that generate many of these structures are also present and active in the blood. While many of these glycosyltransferases are freely circulating in the plasma, we show that platelets are carriers for others, including ST3Gal-1 and β4GalT, and these enzymes are released upon platelet activation. We further show that there are important differences between mouse and human in the abundance of these circulatory glycosyltransferase activities. In particular, human platelets, unlike mouse platelets, are also carriers for large amounts of the circulating ST6Gal-1 and the α3-sialyl to Type-II LacNAc SiaT that can be released upon their activation. Taken together, our results highlight the ability of plasma and activated platelets to supply active glycosyltransferases with the potential to remodel functionally important glycan motifs on the surfaces of target cells.

Results

Differential compartmentalization of circulatory glycosyltransferase activities

To probe the diversity of glycosyltransferases in systemic circulation, we examined the SiaT and FucT activities that act on the three common disaccharide terminal motifs present on cell surface and extracellular glycans, the Type-I and Type-II LacNAc (Gal(β3)GlcNAc, Gal(β4)GlcNAc, respectively), and the mucin-type Core 1 disaccharide (Gal(β3)GalNAc) (Figure 1). The galactosyltransferase (GalT) activities that construct the LacNAc disaccharides from GlcNAc monomers were also examined. Activities in plasma and in sera were comparatively assessed. The plasma activities represent the glycosyltransferases that are extracellular and freely circulating in the blood whereas activities present in sera represent not only the plasma enzymes but also those glycosyltransferases that are released upon blood coagulation. Release of platelet granule content is a major feature of coagulation.

SiaT, FucT and GalT activities were assessed using the traditional, highly sensitive approach of monitoring the transfer of radiolabeled sugars from sugar-nucleotide substrates onto the *O*-benzylated (O-Bn) glycan derivatives. The benzyl group at the reducing terminus permits separation of the radiolabeled products from the unreacted sugar-nucleotides by hydrophobic C-18 solid phase extraction. SiaT products from Type-II LacNAc were further separated into α2,6- and α2,3-sialylated products based on their affinity to SNA, the *Sambucus nigra* lectin. SNA-binding sialyl products were not formed on Type-I LacNAc or mucin-type Core 1 structures (data not shown), and therefore SNA separation was not routinely performed for SiaT on these acceptor substrates.

Figure 2 summarizes the circulatory SiaT (Panel A), FucT (Panel B) and GalT (Panel C) found in mouse plasma and serum. The data confirms a plethora of glycosyltransferases activities in systemic circulation. In general, these glycan-construction blood enzymes appear to be predominantly extracellular, and no differences were observed between plasma and sera activities. There were two notable exceptions; the SiaT against mucin-type Core 1 and the GalT toward GlcNAc that were elevated 2.2-fold and 1.3-fold in serum than in plasma. The FucT activity toward Type-II LacNAc was readily observed in mouse plasma, but barely detectable in serum. The rationale for the disappearance of this FucT activity in serum is not clear at present. FucT activity toward Type-I LacNAc was only marginally detectable, and had a high signal-to-noise ratio making quantification unreliable (data not shown). Therefore, MS^n was used to validate the presence of circulatory enzymes to fucosylate Type-I

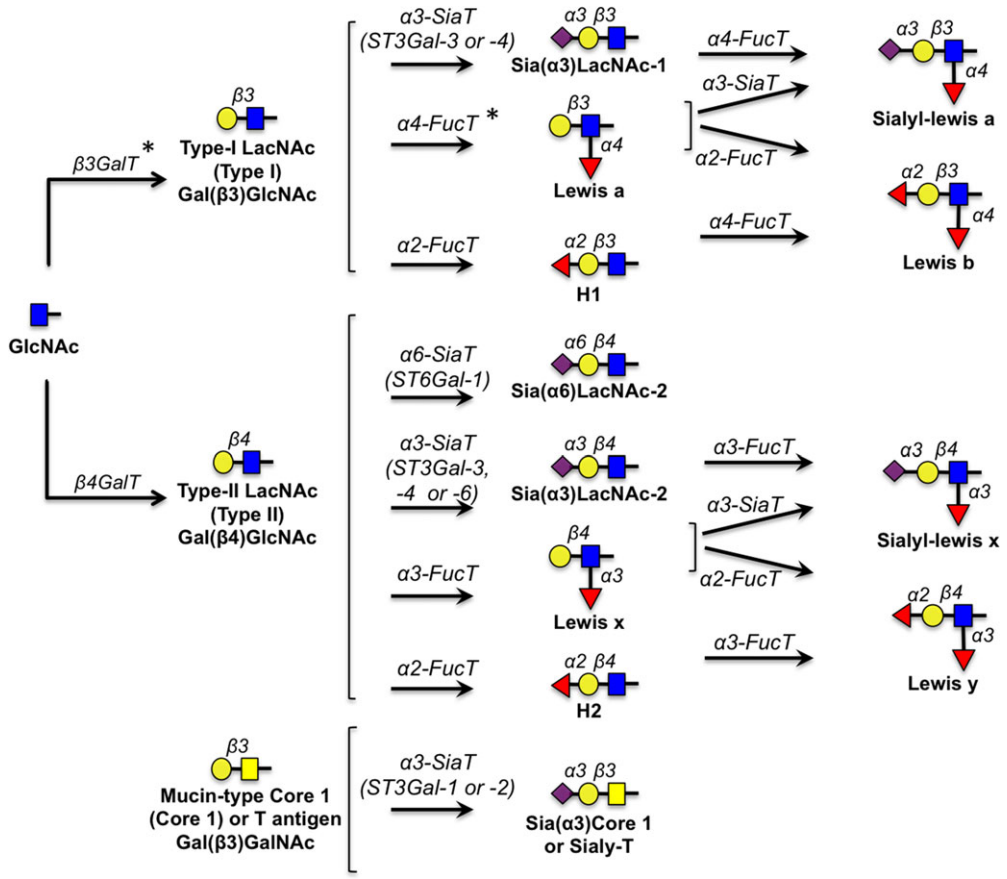

Fig. 1. Schematic diagram of galactosyl modification of GlcNAc and sialyl- and fucosyl- modifications of LacNAc and mucin-type Core 1 disaccharides studied in this paper. Except for those reactions marked by asterisks, extracellular glycosyltransferases that are freely circulating or increased upon platelet activation can construct the glycan structures indicated. This figure is available in black and white in print and in colour at Glycobiology online.

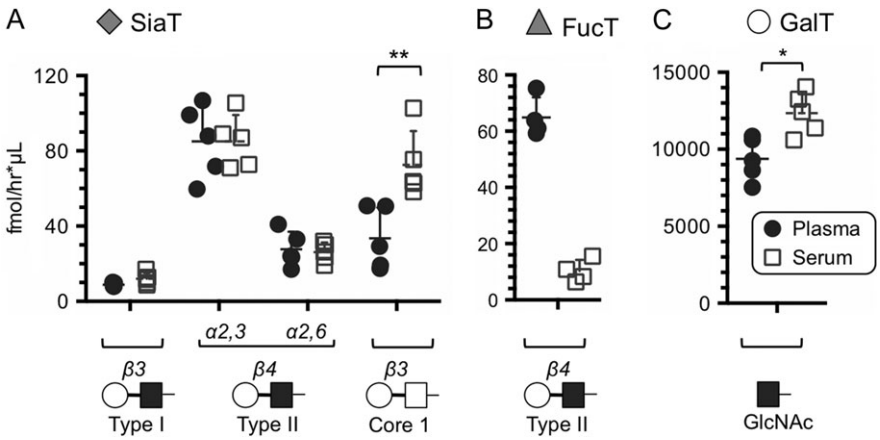

Fig. 2. Activity toward GlcNAc and mucin-type Core 1 acceptor in WT murine serum and plasma. Panels **A**–**C**: Glycosyltransferase activity toward LacNAc, mucin-type Core 1 and GlcNAc acceptors in WT murine plasma and serum. A comparison of glycosyltransferase activity, namely SiaT (**A**), FucT (**B**) and GalT (**C**), in WT murine plasma (filled circle) and serum (unfilled squares) was assessed by measuring the amount of radiolabeled nucleotide-sugar donor transferred various acceptors ($n = 5$). SiaT activity toward Type-II LacNAc, whether displaying α2,3- or α2,6- linkage, was further deconvoluted using agarose- bound SNA lectin. GalT activity toward GlcNAc and SiaT activity toward mucin-type Core 1 acceptor is increased in WT murine serum compared to plasma by 1.3- and 2.2-fold, respectively. SiaT activity toward type-II LacNAc is predominantly α2,3-linked, shown by lectin chromatography. Using MS^n analysis, it was determined that FucT activity in murine plasma and serum toward Type-II LacNAc produces predominantly $Lewis^x$ (Le^x) structures (Figure 3).

LacNAc (see below). The relatively low FucT activity is probably attributed to the LacNAc disaccharides not being an ideal acceptor for activity, rather than the absence of FucT in blood.

Product identification by MS^n

FucTs can mediate the attachment of fucose (Fuc) to either the Gal or the underlying GlcNAc on the LacNAc acceptors to form the Fuc

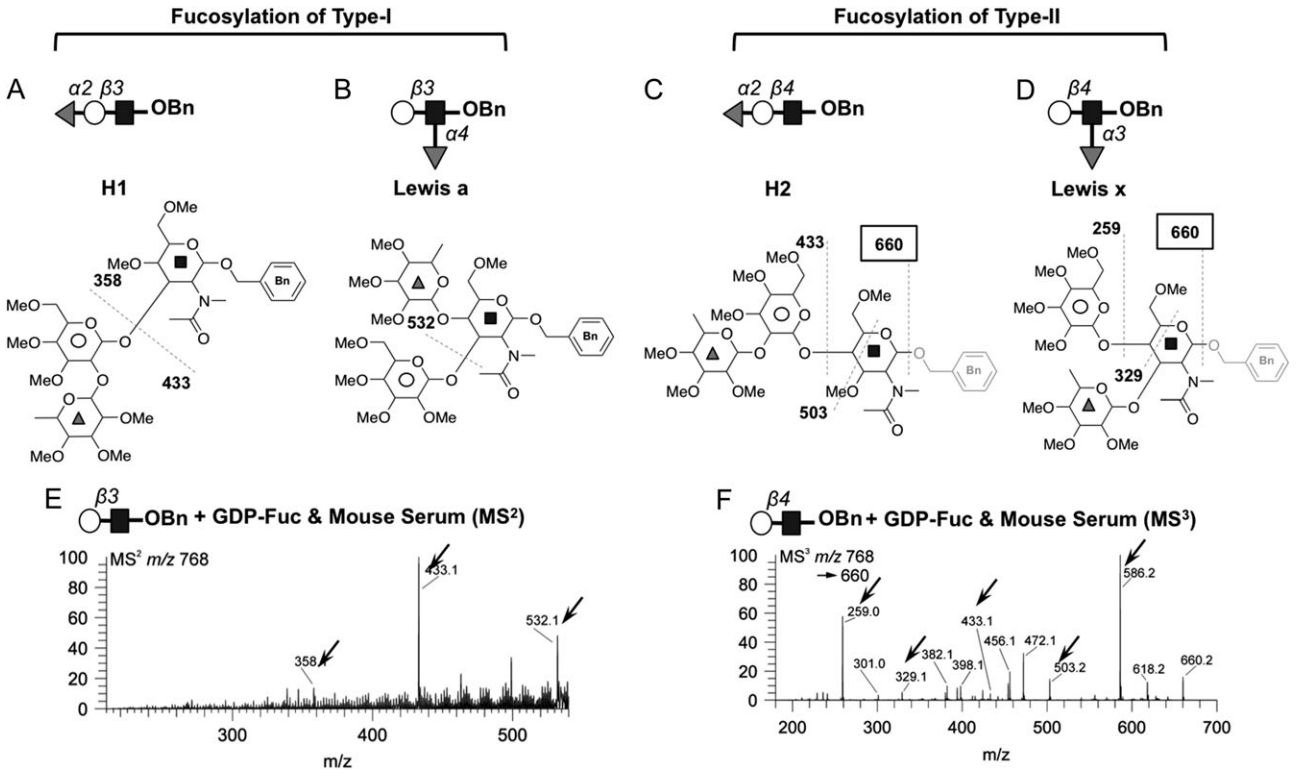

Fig. 3. MS^n determined linkage of glycosylation on fucosylated LacNAc acceptors. Panels **A** and **B** show the structures and representative fragment masses for the fucosylated Type-I LacNAc, H1 and Lewis a, respectively whilst panels **C** and **D** show the structures and representative fragment masses for the fucosylated Type-II acceptor, H2 and Lewis x, respectively. The *m/z* 660 fragment of the latter set is emphasized to indicate that this is the ion fragment analyzed for linkage identification (Panel **F**). Panel **E** shows the fucosylated trisaccharide isolated from the product formed by incubation of a Type-I LacNAc with mouse serum and GDP-Fuc. Panel **F** shows a zoomed in view of the *m/z* 660 fragment of the product formed by incubation of a Type-II LacNAc with mouse serum and GDP-fucose. Both spectra seem to indicate mixtures of the relevant Lewis and H antigen structures. Diagnostic ions are indicated with the arrows.

(α2)Gal(β3/4)GlcNAc (H1 or H2 antigens, respectively) or the Gal (β3/4)[Fuc(α4/3)]GlcNAc (Lewis a or x antigens, respectively). To unequivocally identify the Fuc linkages formed by sera FucT, sequential MS^n was used (Figure 3) (Ashline et al. 2005; Ashline et al. 2014). Fuc addition by sera FucTs to Type-I LacNAc yields both the H1 and the Lewis A antigens, yielding the predicted diagnostic MS^2 fragment ions of *m/z* 433 and 532, respectively, from the parent *m/z* 768 MS^1 ion (Figure 3, Panels A, B and E). Assignment of H1 and Lewis A was confirmed by further MS^3 fragmentation (data not shown). The linkage analysis of the Fuc addition to Type-II LacNAc is summarized in Panels C, D and F. The MS^2 *m/z* 660 fragment yielded MS^3 peaks (indicated by arrows in Panel F) consistent with the presence of H2 and Lewis x based on comparison with the spectra of pure authentic standards (data not shown). Together, the MS^n analysis confirms the ability of mouse circulatory FucT to construct Fuc(α2) to Gal (H1 and H2 antigens) as well as the Fuc(α4/3) to GlcNAc (Lewis a and x) linkages. Human circulatory FucT was also analyzed (Supplementary data, Figure S1) and like mouse blood FucT, has the ability to construct the H1, H2, Lewis a and Lewis x structures.

Figure 4 shows the MS^n analysis to examine the ability of circulatory GalT to generate Gal(β3)GlcNAc and Gal(β4)GlcNAc linkages (i.e. Type-I and Type-II LacNAc) from GlcNAc-O-Bn. The MS^n analysis relies on differing bond labilities of the Gal(β3) or Gal (β4) to GlcNAc linkages, relative to the O-Bn bond to yield diagnostic MS^2 ions, *m/z* 358 and *m/z* 486, respectively, based on analysis of authentic Type-I and Type-II LacNAc-O-Bn standards (Panel C and D, respectively) (Ashline et al. 2014). When Type-I LacNAc is formed, the Galβ(3)-linkage is more labile than the O-Bn bond and cleavage products of the former dominate its MS^2 spectrum. On the other hand, the O-Bn bond of Type-II LacNAc is weaker than the Gal(β4) linkage and the Gal(β4)GlcNAc fragment dominates the spectrum, although Gal(β4)GlcNAc cleavage products are detectable at low abundance. When MS^2 was performed on the products formed by mouse sera on GlcNAc-O-Bn, only the *m/z* 486 ion, but not the 358 ion was present (Panel E). To further clarify the nature of the galactosyl linkage formed by circulating mouse enzymes, MS^3 was performed on the *m/z* 486 ion (Panel F) to look for the presence of the 3,5A cross ring cleavage (*m/z* 329) found in Type-II LacNAc spectra. Thus, the data indicate the ability of circulatory mouse GalT to assemble Type II-LacNAc-O-Bn (i.e. β4GalT), but not Type I-LacNAc-O-Bn (i.e. β3GalT) from GlcNAc-O-Bn.

The MS^n approach was also used to corroborate the assignment of α6- and α3-sialyl linkages generated on Type-II LacNAc-O-Bn done by utilizing specific affinity of SNA towards α6-sialyl structures (Supplementary data, Figure S2) (Ashline et al. 2014). Serum sialylated Type II-LacNAc-O-Bn products were fragmented in the ion trap to generate the Gal(β4)GlcNAc fragment (*m/z* 472). The resulting spectra were compared to those of the same fragment isolated from pure α6 and α3 standards, which were generated using purified recombinant ST6Gal-1 acting on Type II-LacNAc-O-Bn or from the Consortium for Functional Glycomics, respectively. The purified recombinant enzyme generated sialyl-standards were also used to ensure the specificity of the SNA separation in the radioactive assays for α6- but not α3-sialyl products (data not shown). Both α6- and α3-sialyl standards showed common fragmentation ions at *m/z* 227,

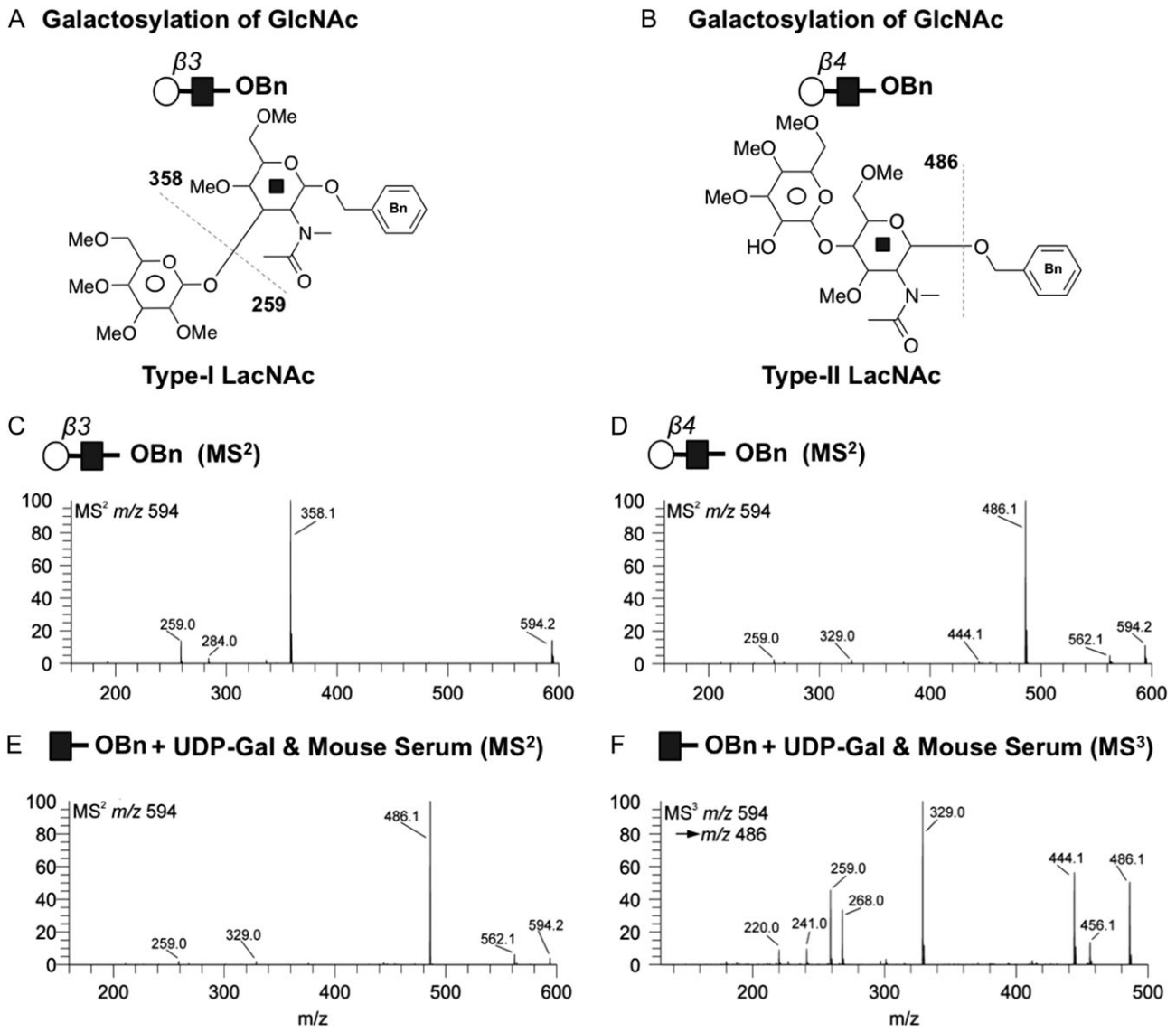

Fig. 4. MS^n for spectral identification of galactosylated GlcNAc products. Panels **A** and **B** show the Type-I (Gal(β3)GlcNAc-O-Bn) and Type-II (Gal(β4)GlcNAc-O-Bn) LacNAc, respectively, with fragment masses identified. Panels **C** and **D** show the MS2 spectra of the Type-I and Type-II standard materials, respectively. Panel **E** shows the MS2 spectrum of the galactosylated product isolated from the reaction mixture. The pattern of fragments matches the spectrum of the Type-II standard. Panel **F** shows the MS3 spectrum of the *m/z* 486 fragment, empirically showing the β4-linkage of the product. Mass spectral identification of the Type-I and Type-II LacNAc relies on the differing bond labilities of the Gal(β3)- and Gal(β4)GlcNAc linkages relative to the *O*-benzyl bond. For Type-I acceptor, the Gal-β1,3 linkage is relatively more labile and thus its MS^2 spectrum is dominated by products of that cleavage. For Type-II LacNAc, the *O*-benzyl bond is weaker than the Gal-β1,4 linkage and the B-type Gal-GlcNAc fragment dominates the spectrum, though Gal-GlcNAc cleavage products are detectable at low abundance. Identification of the unknown is based on a simple comparison of the obtained spectrum with those of the standards. Further clarification of the linkage can be made by the presence of the cross ring cleavage (*m/z* 329) found in the Type-II LacNAc spectra as well as those of the GalT reaction product.

268 and 315 (arrows), but the relative intensities of these signature peaks consistently differ between the α3- and α6-sialylated standards. The resulting spectrum produced by serum enzymes had a pattern that was intermediate between the pure standards and thus is consistent with a mixture of α3- and α6-linked sialic acids to Type II-LacNAc were constructed by serum SiaT.

Platelets are major carriers of circulatory ST3Gal-1 in the mouse

In order to understand the source of the SiaT toward the mucin-type Core 1 structure released upon preparation of sera, we hypothesized that this SiaT activity is platelet-associated and released upon activation of the coagulation cascade. Studies from Hoffmeister and Hinsdale have shown that platelets are a direct source of many glycosyltransferases, especially of β4GalT, that we have shown to be also elevated in sera, relative to plasma (Figure 2) (Condac et al. 2009; Wandall et al. 2012). To test this hypothesis, SiaT acting on mucin-type Core 1-O-Bn was comparatively examined in sera and plasma from wild-type (WT) mice and from two mice models with platelet deficiency generated by completely independent means (Figure 5, Panel A). One animal model is the ST3Gal4-deficient mouse ($ST3GalIV^{-/-}$) which has a 3- to 4-fold reduction in circulatory platelet count due to enhanced clearance of the ST3Gal4-deficient platelets by the hepatic asialoglycoprotein

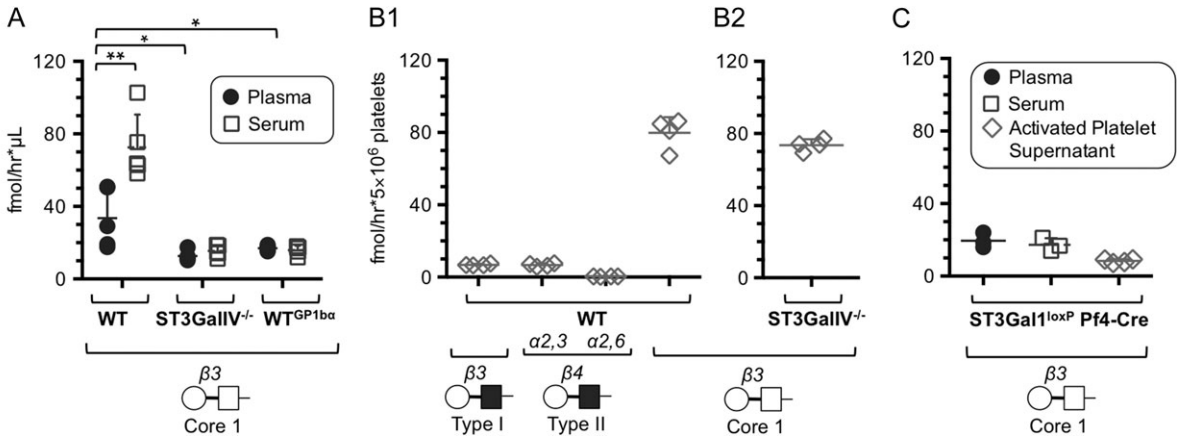

Fig. 5. Increased activity toward mucin-type Core 1 is attributed to ST3Gal1 released by activated platelets. Panel **A**: Mouse models displaying low-platelet count did not show observed increase in serum SiaT activity toward Type-III acceptor. The difference plasma (filled circles) and serum (unfilled squares) SiaT activity in WT mouse is compared to two thrombocytopenic mouse models (ST3GalIV$^{-/-}$ and WT$^{GP1b\text{-}alpha}$). SiaT activity toward mucin-type Core 1 was not differentiated in plasma (filled circle) and serum (unfilled square) in the two thrombocytopenic mouse models. Additionally, levels of SiaT active toward mucin-type Core 1 in plasma were lowered in thrombocytopenic mouse models compared to WT mice. Panel **B**: Increased activity toward mucin-type Core 1 is contributed directly by platelets. The supernatant of activated isolated platelets were examined for SiaT activity (pooled platelets, $n = 4$). Each µL in the assay contained supernatant from approximately 5×10^6 of platelets, activated with thrombin. Both activated WT and ST3GalIV$^{-/-}$ platelet supernatant contains equally active SiaT active toward mucin-type Core 1, showing that the platelets directly supply SiaT active toward mucin-type Core 1 and that lowered serum activity observed in ST3GalIV$^{-/-}$ is due to low-platelet count in the system, and not a SiaT deficiency within the platelets. No observable transfer of [H3]-NeuNAc toward Type-I and Type-II LacNAc by activated WT platelet supernatant was observed. Panel **C**: Lowered activity of plasma, serum and platelet supernatant from ST3Gal1^{loxP} Pf4-Cre mice show that the increased SiaT activity toward mucin-type Core 1 is attributed to ST3Gal1. ST3Gal1^{loxP} Pf4-Cre plasma and serum show no difference in SiaT activity toward mucin-type Core 1 ($n = 3$). Supernatant of activated isolated ST3Gal1^{loxP} Pf4-Cre platelets (unfilled diamonds) show no activity toward mucin-type Core 1 (unpooled platelets, $n = 4$).

receptor (Ellies et al. 2002). For the second model, circulatory platelets were depleted 6-fold from normal levels in WT animals by injection of anti-GP1bα (WT$^{GP1b\text{-}alpha}$) (Li et al. 2006). Both ST3GalIV$^{-/-}$ and WT$^{GP1b\text{-}alpha}$ models had strikingly diminished SiaT against mucin-type Core 1 in both serum and plasma fractions (Figure 5, Panel A). Furthermore, when comparing just the plasma of the WT mouse with both the platelet-depleted models, SiaT activities only toward mucin-type Core 1 is decreased 2.6-fold and 1.9-fold in the ST3GalIV$^{-/-}$ and WT$^{GP1b\text{-}alpha}$ models, respectively (Figure 5, Panel A). This indicates that thrombocytopenic conditions can affect the level of the SiaT even prior to platelet activation and coagulation events. Taken together, these observations suggest that platelet activation and coagulation events may dramatically alter the amount of freely circulating active SiaT.

To demonstrate conclusively that platelets are the principal carriers of circulatory SiaT against mucin-type Core 1, platelets isolated from WT animals were activated in vitro by thrombin. Supernatant from *in vitro* activated platelets had SiaT activity only toward mucin-type Core-1, but not Type-I or Type-II LacNAc acceptors (Figure 5, Panel B1). The measured SiaT activity toward mucin-type Core-1 from platelets was 80 fmol/hr/5×10^6 platelets. Given that normal platelet counts in circulation are around 1 to 2×10^6 platelets/µL of blood, the level of SiaT activity toward mucin-type Core 1 released by activated platelets was roughly equivalent to that found in sera. Moreover, platelets isolated from the ST3GalIV$^{-/-}$ animals released the same level of SiaT activity toward mucin-type Core 1 structures as WT platelets (Figure 6, Panel B2), ruling out the remote possibility that ST3Gal4 deficiency directly caused the lack of SiaT activity toward mucin-type Core 1.

Two SiaT, ST3Gal-1 and ST3Gal-2, have the ability to sialylate mucin-type Core 1 (Harduin-Lepers et al. 2001). In order to distinguish which of these two SiaT are carried by platelets, a third animal model, the ST3Gal1^{loxP} Pf4-Cre was examined. The ST3Gal1^{loxP} Pf4-Cre mouse is ST3Gal-1 deficient only in the megakaryocyte (MK)-platelet lineage. As shown in Figure 5, Panel C, SiaT activity towards mucin-type Core 1 were strikingly reduced in both serum and plasma, and especially low in activated platelets. Thus, together the data indicate that ST3Gal-1 in circulation is principally carried by platelets. Therefore, platelet ST3Gal-1 accounts for much of the circulatory SiaT activity toward mucin-type Core 1, putatively generating the extension structure, the Sia(α3)Gal(β4)GalNAc, common on O-linked glycans.

Human platelets and glycosyltransferase activities

The constituent glycosyltransferase activities present in human platelets are profoundly different than those in mouse platelets, despite essentially similar profiles in plasma and serum between the two species. Human platelets from three healthy volunteers were freshly isolated and activated in vitro. The glycosyltransferase activities harbored by the platelets or released upon platelet activation were compared to fresh mouse platelets activated by a comparable procedure, and the results presented in Figure 7. Mouse platelets contained negligible levels of most SiaT, except for the SiaT to mucin-type Core 1, identified in the mouse as ST3Gal-1. Human platelets have strikingly high SiaT activities, both α3- and α6- to Type-II LacNAc, both of which are released upon platelet activation. In contrast, the corresponding SiaT activities are essentially absent in mouse platelets (Figure 7, Panels A and B). GalT to GlcNAc acceptor activity is also 10-fold higher in human, compared to mouse platelets (Figure 7, Panel C). Interestingly, both mouse and human platelets construct both Gal(β3) and Gal(β4)GlcNAc linkages, observed via MS^n, whereas the latter is only observed in circulatory blood. Thus, both mouse and human platelets contain a store of β3GalT not observed in circulation, possibly suggesting that β3GalT is a cell-associated enzyme that is not released freely as a soluble entity.

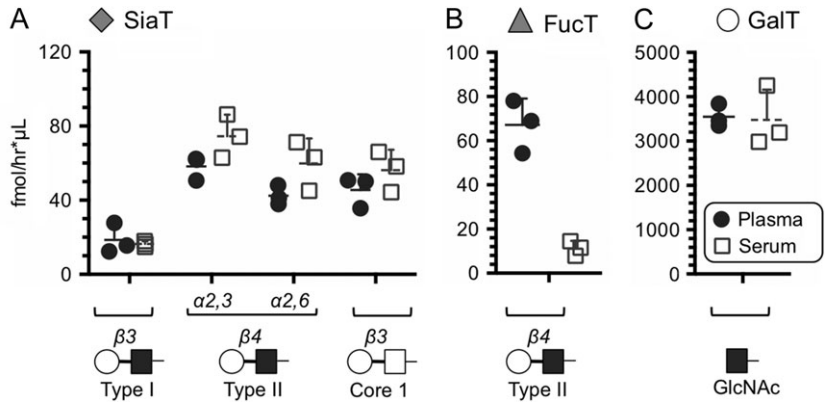

Fig. 6. Comparing glycosyltransferase activity in healthy human plasma and serum samples to murine samples. Panels **A**–**C**: No significant difference between plasma and serum glycosyltransferase activities were observed in human samples. A comparison of the glycosyltransferase activity, namely GalT (**A**), SiaT (**B**) and FucT (**C**), in healthy human plasma (filled circle) and serum (unfilled squares) was assessed by measuring the amount of radiolabeled nucleotide-sugar donor transferred to GlcNAc and LacNAc acceptors ($n = 3$). In human compared to mouse, increased SiaT activity toward Type-II, particularly α2,6-sialylation, and mucin-type Core 1 acceptors are observed. Additionally, SiaT activity toward mucin-type Core 1 is also increased in human plasma compared to murine plasma; however, serum levels of the same SiaT activity are comparable between human and murine systems. FucT activity toward LacNAc acceptors is ~2-fold higher in human circulation than murine whereas GalT activity is ~5-fold lower in human circulation compared to murine systems. Additionally, fucosylation of Type-II acceptor, though higher in mice, seems to contain equal mixtures of both Le^x and H2 structures, unlike the murine blood, which had dominantly Le^x structures (MS^n analysis, Supplementary data, Figure S1).

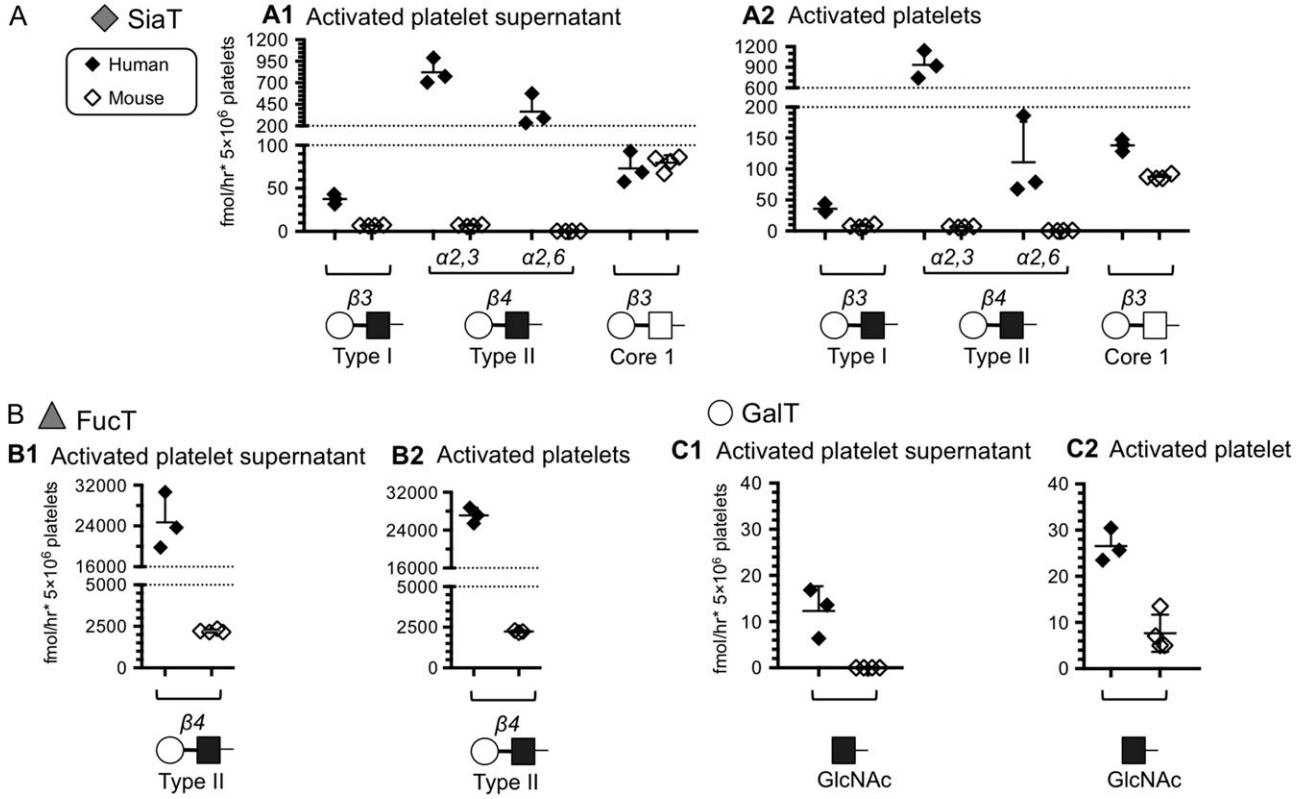

Fig. 7. Comparing glycosyltransferase activity between human and murine platelets. Panels **A**: SiaT activity. Panels **B**: GalT activity. Panels (**A** and **B**)1: Activated platelet supernatant. Panels (**A** and **B**)2: Activated platelets. Human (filled diamond, $n = 3$, unpooled), Mouse (unfilled diamond, $n = 4$ or 5, pooled). Human platelets contain more glycosyltransferase activity than mouse platelets. When activated, mouse platelets contain GalT and only the SiaT active toward mucin-type core 1 structure. Human platelets also release the same glycosyltransferases, and additionally have higher SiaT activity toward Type-II acceptor, particularly ST6Gal-1, which creates an α-2,6 linkage to the acceptor. Panel **C1**: FucT activities in human and mouse platelets toward LacNAc disaccharides. Mouse and human platelets fucosylate Type-II LacNAc with different ratios of Lewis x and H2 structures. Type-I LacNAc fucosylation by mouse platelets is not detected whether by radiolabel- or mass spectrometry-based assays and is barely discernible for fucosylation by human platelets.

Human platelet FucT activities were low but detectably greater than corresponding activities in mouse platelets (Figure 7, Panel C1). Mass spectrometric product analysis confirms the ability of human platelets to construct the H1, H2, as well as Lewis A and Lewis X structures.

Human circulatory SiaT, FucT and GalT against LacNAc

Mouse is the animal experimental model of choice for human conditions. However, important differences may exist between human and mouse. Therefore, plasma and serum from healthy human volunteers were also assessed for the same panel of glycosyltransferase activities (Figure 6). The pattern of glycosyltransferases in human blood is essentially similar to those in mouse, but with a few prominent differences. ST6Gal-1, identified as the SiaT constructing the α6-sialyl to Type-II LacNAc, was 2- to 3-fold more abundant in human systemic circulation than in mouse blood. GalT was also strikingly higher (5-fold) in human compared to mouse blood (compare Figure 6 to Figure 2). Additional differences were also revealed by comparative MS^n product analysis of the glycosyltransferase activities. While mouse blood produced mostly fucosylated Le^x (Figure 3, Panel F), human blood produced a roughly equal mixture of fucosylated Le^x and H2 (Supplementary data, Figure S1). Finally, in the samples from all three human volunteers, there was no difference between plasma and serum in GalT and in SiaT to mucin-type Core 1 activities, as had been observed in the murine samples.

Discussion

The idea that a variety of glycosyltransferase activities are present in the blood has been known for several decades. However, as a group, blood-borne glycosyltransferases have remained poorly characterized due to a lack of understanding for their physiologic function in the blood. In the present report, we demonstrated that many of the well-recognized and biologically relevant glycan motifs could be constructed by blood-borne glycosyltransferases. These motifs include many of the sialyl-Lewis structures that are the typical ligands for selectin-mediated cell adhesion (Kannagi et al. 2004; Sperandio et al. 2009) as well as ligands for the Siglec family of receptors (Varki and Angata 2006). Conversely, fucosylation of Type II LacNAc to form Lewis x on leukocytes can attenuate leukocyte recruitment to the endothelial upon inflammation by disrupting integrin-mediated interactions (Buffone et al. 2016). Modification of the LacNAc and Core 1 motifs may also alter the recognition of these structures by the galectins, another family of carbohydrate binding receptors with established roles in cell differentiation, proliferation, death and malignancies (Garner and Baum 2008; Boscher et al. 2011; Dimitroff 2015; Thijssen et al. 2015).

Glycans, synthesized by glycosyltransferases, occupy the interface between each cell and its external environment. In the canonical glycosylation process, glycosyltransferases are compartmentalized within the ER-Golgi secretory apparatus where they assemble glycans on neo-glycoproteins and glycolipids in transit. Blood-borne glycosyltransferases are extracellular enzymes. These extracellular enzymes, which we will now refer to as "extrinsic glycosyltransferases", have the potential to modulate critical cell–niche interactions by remodeling the glycan architecture of target cell surfaces (i.e. extrinsic glycosylation). In this report, we provide confirmation that a plethora of glycosyltransferases, including members of the SiaT, GalT and FucT families, exist in blood and with the potential to mediate extrinsic glycosylation as diagrammed in Figure 1. Previously, we demonstrated that activated platelets are a functional source of CMP-Sia sugar-donor driving extrinsic sialylation (Lee et al. 2014). Others have shown that platelets also contain a cache of other sugar precursor substrates, such as UDP-GalNAc, UDP-Gal, which is released into circulation upon platelet activation (Wandall et al. 2012; Jones et al. 2016). It still remains to be demonstrated that platelets can likewise drive extrinsic fucosylation or galactosylation events. Thus, it is likely that cell surface glycans are being constantly remodeled in circulation by blood-borne glycosyltransferases, with platelets being the crucial regulator of this activity.

Circulatory glycosyltransferases have the potential to impact a wide variety of pathologic processes. Extrinsic ST6Gal-1 in systemic circulation is an attenuator of marrow hematopoietic activity. In all mammals, the systemic inflammatory response, i.e. the acute phase reaction, is accompanied by 3- to 4-fold elevation in plasma ST6Gal-1 (Kaplan et al. 1983; Lammers and Jamieson 1986; Wang et al. 1989; Dalziel et al. 1999). An important physiologic aspect of the acute phase reaction is to limit the inflammatory response to the site of injury and a return to homeostasis. Likely, circulatory ST6Gal-1 contributes to this process by suppressing production of new inflammatory cells (Nasirikenari et al. 2006). Indeed, circulatory ST6Gal-1 deficiency is associated with a generally pro-inflammatory state (Nasirikenari et al. 2010). Our current understanding, based on mouse modeling, is that most of the blood ST6Gal-1 is produced in the liver (Appenheimer et al. 2003) where its expression is regulated by circulatory glucocorticoids (van Dijk et al. 1986; Wang et al. 1990) and cytokines (Woloski et al. 1985; Jamieson et al. 1987), including IL-6 (Dalziel et al. 1999). Furthermore, the idea that serum levels of glycosyltransferases, especially the circulatory ST6Gal-1, as indicators of cancers and their prognosis has long been proposed and supported by multiple studies (Dall'Olio 2000; Dall'Olio and Chiricolo 2001). Interestingly, human, but not mouse platelets, have a large cache of compartmentalized ST6Gal-1 (see Figure 7, Panel A). Given the role of circulating ST6Gal-1 in hematopoietic regulation, it is noteworthy that a significant amount of circulatory ST6Gal-1 in humans is of non-hepatic origin, and unlikely to be regulated by the same mechanism governing liver ST6Gal-1. Therefore, it is likely that in humans but not in mouse, platelet ST6Gal-1 contributes to the overall pool of circulating ST6Gal-1, in addition to its partner sugar-donor substrate CMP-Sia, upon activation of the coagulation cascade.

Various cancers and malignancies, of hematological origin or otherwise, are associated with changes in platelet count. Thrombocytosis occurs in 10–50% of patients with solid malignancies and is associated with significantly shorter survival times (Buergy et al. 2012; Yuan and Xishi 2015). Ovarian cancers that produce the cytokine IL-6, driving platelet production by stimulating production of hepatic thrombopoietin, have been correlated with poorer outcomes (Stone et al. 2012). This implies a distinct possibility that malignancy-induced changes in platelet homeostasis and activation can contribute to the overall tumor microenvironment whereby extrinsic remodeling of cell surface glycans alters the host anti-tumor response. Indeed, removal of sialic acids from immune cell surfaces such as dendritic cells results in their enhanced ability for anti-tumor activity (Silva et al. 2016). By inference then, excessive sialylation, triggered by platelet activation, should impose a dampening of normal immune cell functions, leading to poorer outcomes in malignancies with thrombocytosis. Perhaps, it is not a coincidence that hepatic production of both ST6Gal-1 and thrombopoietin are driven through activation of the IL-6 receptor under inflammatory or malignant conditions.

The comparison of glycosyltransferase activities between human and mouse blood revealed a number of pronounced differences, mostly related to platelets. While ST3Gal-1 (α3-sialyl specific to Core 1) is equally active in both human and mouse platelets, human platelets are 10-fold more active for the β4GalT. Moreover, human platelets are strikingly enriched for both α3- and α6-SiaT activities towards Type-II LacNAc, both of which are conspicuously absent in mouse platelets. The presence of additional and unique glycosylation capability in human platelets suggests physiologic and functional divergence between human and mouse platelets. For example, sialylation of LacNAc by ST3Gal4 is a major parameter for persistence of platelets in circulation (Ellies et al. 2002; Sorensen et al. 2009). Inactivation of ST3Gal4 in mice led to pronounced thrombocytopenia due to rapid clearance via the hepatic Ashwell–Morell Receptor (Rumjantseva et al. 2009). Human platelets have an average circulatory half-life of 7–10 days, which is substantially longer than the average half-life of mouse platelets of only 4–5 days. This could be possibly due to the significant cargo of ST3Gal4 and β4GalT residing in human platelets but not mouse platelets. The ability of platelets to secrete GTs and, perhaps, glycosidases supports the concept that they are used to reversibly glycosylate glycoproteins, possibly regulating the lifespan of platelets. These enzymes, which are also present in the blood, can assemble the sialyltrisaccharide precursor for the selectin receptor ligand, sialyl-Lewis X. Thus, human platelets have the tantalizing potential to be regulators of inflammation by generating critical ligands for selectin-mediated cellular adhesive events.

The mouse has been an important model organism to study and to evaluate human normal and disease processes. The prominent differences in the abundance and distribution of the blood-borne glycosyltransferases, however, further illustrate the need to validate in humans any information gathered using the mouse or any other species as models. Furthermore, the data presented here reveal that the platelets are a major transporter of blood-borne glycosyltransferases. This implicates the possibility of a platelet glycosylation axis in regulating hematopoietic homeostasis and in generating glycan ligands for cell trafficking and in inflammation, driven chiefly by signaling through hepatocytes.

Materials and methods

Animals and plasma/serum collection

All mouse strains are C57BL/6, WT and ST3GalIV$^{-/-}$ mice as described (Ellies et al. 2002). ST3Gal1^{loxP} mice (Priatel et al. 2000) were paired with Pf4-Cre mice (The Jackson Laboratory) to inactivate the ST3Gal1 gene in the MK lineage (Tiedt et al. 2007). The ST3Gal1^{loxP} PF4-Cre males obtained were viable, fertile, and further paired with ST3Gal1^{loxP} females. Thus, the ST3Gal1^{loxP} Pf4-Cre mice are ST3Gal-1 deficient only in the MK-platelet lineage. Murine plasma and serum were collected via retro-orbital eye-bleeds. Additionally, plasma was collected in K_2-EDTA tubes and processed immediately whereas blood for serum collection was allowed to coagulate for 1–2 h. Thrombocytopenia was induced in WT mice by injection of 1 μg/g of GP1bα antibody introduced via tail-vein (Li et al. 2006). Plasma and serum collection from these mice were performed 2 h after injection. The collected blood was spun at 16,000 × *g* for 5 min to form cell pellets. The supernatant stored in multiple aliquots at −80°C to ensure that samples underwent only one freeze/thaw cycle for the glycosyltransferase assay. The Institute Animal Care and Use Committee of Roswell Park Cancer Institute have approved all animal studies presented here. Approval for human blood drawing was obtained from the institutional review board of Brigham and Women's Hospital, and informed consent was obtained according to the Declaration of Helsinki.

Platelet collection and activation

Murine or human blood was collected in ~0.1 volume of K_2-EDTA or sodium citrate anticoagulant (Sigma-Aldrich, MO) and centrifuged at 200 × *g* to obtain platelet-rich plasma. Platelets were separated from plasma proteins by centrifugation at 900 × *g* (Hoffmeister et al. 2003). After separation from plasma, platelets were washed as described (Wandall et al. 2012). Platelets were activated by addition of 0.2 U of human thrombin (Sigma-Aldrich) at 37°C for 5 min. The concentration of platelets was determined via flow cytometry by using 5.5 μm of SPHERO^{TM} Rainbow Fluorescent Particles (Spherotech, IL) as a reference.

Glycosyltransferase assays

The four *O*-benzyl derivatized acceptor structures were used; GlcNAcα-O-Bn, Type-I LacNAc (Gal(β3)GlcNAcα-O-Bn), Type-II LacNAc (Gal(β4)GlcNAcα-O-Bn) and mucin-type Core-1 (Gal(β3) GalNAcα-O-Bn), obtained from Toronto Research Chemicals. Three sugar-nucleotide donors were used as well: for radioisotope-based assays, CMP-[^{3}H]-NeuAc, UDP-[^{3}H]-Gal (both from American Radiolabeled Chemicals, MO) and GDP-[^{14}C]-Fuc (PerkinElmer, MA) were used. Assays analyzed by MS^n used the same set of non-radiolabeled sugar nucleotides (Sigma-Aldrich). The incubation mixture (3 μL) contained a buffer mixture with 2.5 mM acceptor, 1.25 μM radiolabeled or 0.1 mM non-radiolabeled sugar-nucleotide donor, 5 μg/μL BSA and 0.5% (W/V) Triton X-100. Addition of BSA and Triton X-100 was omitted in the assays used in mass spectrometry analysis. SiaT assays were buffered with 50 mM sodium cacodylate pH 6.5 (Chandrasekaran et al. 1995) whereas GalT and FucT activities were performed in 25 mM Tris-HCl pH 7.5 supplemented with 1 mM each of $MnCl_2$ and $MgCl_2$. Incubation was carried out at 37°C for 1.5 h. Transfer of radioactively labeled sugars to benzyl derivatized acceptors was monitored by scintillation counting after removal of unreacted radiolabeled sugar-nucleotides and by-products by C18-reverse phase chromatography (SepPak) (Ujita et al. 1998; Appenheimer et al. 2003). Separation of α2,3- and α2,6-sialic acid fractions of sialylated Type-II LacNAc was performed with agarose-bound SNA (*S. nigra agglutnin)*-lectin (Vector Laboratories, CA) affinity chromatography (Jones et al. 2010).

MS^n of glycosylated O-Bn acceptors

Acceptors were eluted from the glycosyltransferase assay by C18 solid phase extraction (Appenheimer et al. 2003). The O-Bn acceptors were permethylated using a modified spin-column strategy (Desantos-Garcia et al. 2011). Sodium hydroxide beads and iodomethane were from Sigma. Purification of permethylated oligosaccharides was by liquid–liquid extraction with dichloromethane and 0.5 M aqueous NaCl. Permethylated oligosaccharide samples were dissolved in 1:1 methanol/water. Samples were loaded onto a Triversa Nanomate (Advion, NY) mounted to an ion trap mass spectrometer (LTQ, ThermoFisher, CA). Activation Q and activation time were left at the default values, 0.250 and 30 ms, respectively. Collision energy was set to 35% for all CID spectra. Scan rate was set to "Enhanced" for most spectra. Data were acquired in profile mode; all spectra shown are profile mode. MS^n peaks were

selected manually. Signal was averaged from a variable number of scans. Microscan count, AGC target values, and maximum injection time were varied, depending on sample amount and signal intensity. All ions are sodium adducts.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement

None declared.

Abbreviations

Fuc, Fucose; FucT, Fucosyltransferase; GlcNAc, *N*-acetylglucosamine; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GalT, Galactosyltransferase; LacNAc, lactosamine; MS^n, tandem mass spectrometry; SiaT, Sialyltransferase.

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