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Variation of fibrinogen oligosaccharide structure in the acute phase response: Possible haemorrhagic implications

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ABSTRACT

Background: Fibrinogen is an acute phase glycoprotein whose concentration increases in response to trauma. The newly synthesised protein is functionally enhanced and it is known that treatment with neuraminidase increases the rate of fibrin polymerisation. To explore this, we examined the differences between the oligosaccharide structures of quiescent and acute phase fibrinogen.

Methods: A series of plasma samples was obtained from two individuals suffering an acute phase response. Fibrinogen chains were examined directly by ESI mass spectrometry before and after digestion with N-glycosidase F and β1,4 galactosidase.

Results: The Bβ and γ chains of acute phase fibrinogen showed a mass decrease of 162 Da (Gal) in some 50% of the molecules, and the Bβ chain showed an additional decrease corresponding to a further loss of NAcGlc. Incubation with N-glycosidase F normalised all isoform masses to that of the quiescent naked protein, confirming the N-linked oligosaccharide as the source of heterogeneity. β1,4 galactosidase treatment showed the structural difference was the absence of the penultimate Gal from the biantennary oligosaccharides, and mapping of tryptic glycopeptides confirmed these results showing that approximately half the chains lacked Gal.

Conclusions and implications: The failure of incorporation Gal excludes the possibility of the hepatic NAcNeu Gal transferase capping the oligosaccharides with sialic acid. This has two desirable haemostatic outcomes: fibrin monomers will polymerise and form clots more rapidly, and two galactose residues can never be exposed diminishing uptake of the protein by the asialoglycoprotein receptor and ramping up concentration at a time of challenge.

1. Introduction

Fibrinogen is an acute phase glycoprotein that on activation by thrombin polymerises to form a mesh that binds activated platelets and stems blood loss from sites of injury. Correct clot formation and ultimate fibrin dissolution (fibrinolysis) are quite literally matters of life or death. Fibrinogen, a 340 kDa glycoprotein, is synthesised in the liver from individual Aα, Bβ and γ chains [1]. Initially, a signal peptide directs the nascent chains into the endoplasmic reticulum where core glycosylation occurs. Following cleavage of the leader sequence, the mature protein is assembled from Aα-γ and Bβ-γ intermediates [2]. After acquiring an additional Bβ or Aα chain, Aα-Bβ-γ half-molecules dimerise and the $(Aα\text{-}Bβ\text{-}γ)_2$ assembly proceeds to the Golgi for pruning and extension of its N-linked oligosaccharides before final secretion into the circulation.

The Bβ and γ chains each contain a single N-linked oligosaccharide side chain attached to Asn364 and Asn52, respectively. These structures, $\text{-NAcGlc}_2\text{-Man-(Man-NAcGlc-Gal-NAcNeu)}_2$, are exclusively biantennary and terminate with sialic acid (NAcNeu) [3,4]. This means each 340 kDa fibrinogen molecule can have a maximum of eight sialic acids; however, partial in vivo desialylation ensures an average of only ~5 mol/mol of circulating fibrinogen [5]. The precise configuration of the terminal sugars is however important in determining the fibrin polymerisation rate and the circulatory half-life of fibrinogen. Electrostatic repulsion from negatively charged sialic acid residues delays fibrin polymerisation and clot formation [6], and exposure of galactose terminals appears to initiate uptake by the asialoglycoprotein receptor [7] as no circulating fibrinogen molecules have been ever found that lack both sialic acid residues [8]. Different physiological stresses are known to modulate the carbohydrate structure of fibrinogen. In liver disease, increased sialic acid content extends thrombin clotting time [6], and during pregnancy [9], where there is an increased risk of haemorrhage, sialic acid saturation decreases shortening the clotting time.

The acute phase response has a profound effect not only on the concentration but also on the structure of plasma proteins. For example, in the case of α_1 antitrypsin, another liver derived glycoprotein [10], there is a shift to more highly branched triantennary structures, thereby

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increasing its sialic acid content. In the case of fibrinogen, acute phase plasma has been shown to contain more high-molecular weight (HMW) fibrinogen (340 kDa) than LMW fibrinogen (305 kDa) with one of its αC domain cleaved off [11]. The increase in the proportion of HMW fibrinogen accompanying an acute phase is however a consequence of the increased rate of fibrinogen synthesis. Since this α chain cleavage occurs in circulation, LMW fibrinogen simply represents an older population of molecules. Similarly, the higher level of Aα chain phosphorylation associated with an inflammatory response [12] reflects larger numbers of newer molecules in the plasma pool [13].

Bleeding after surgery is a serious complication associated with increased morbidity and mortality [14]. The acute phase response causes endogenous fibrinogen levels to typically double 24 h after surgery [15]. The functionality of this newly synthesised fibrinogen is enhanced as evidenced by the increase seen in the ratio of functional (Clauss) to physical (total clottable) fibrinogen [15]; hence, the acute phase not only produces more fibrinogen but it is better configured for its function. Because of the inherent importance of the carbohydrate structure to the function and concentration of plasma proteins [16], we decided to determine the oligosaccharide structures of fibrinogen molecules synthesised during an acute phase response and compare them to quiescent state structures.

2. Materials and methods

2.1. Protein purification and analysis

Fibrinogen was purified by ammonium sulphate precipitation [17] from Li heparin plasma that had been used for acute phase assessment of C reactive protein (CrP). After reduction (4 h at 37 °C) in 8 M urea, 0.1 M Tris/HCl pH 8, 15 mM dithiothreitol individual fibrinogen chains were isolated by reverse phase HPLC on a Jupiter C-4 (25 × 0.46 cm) column (Phenomenex) [8,17]. Approximately 150 μg of fibrinogen was injected, and ~200 μl of individual Bβ and γ peak crests was collected and 20 μl directly injected into a VG Platform quadrupole analyser at 10 μl/min. The probe was charged at +3000 V, and the electrospray source was maintained at 60 °C. The mass range 1050–1600 m/z was scanned with a cone ramp of 50–70. Up to 120 scans were averaged and transformed using Mass-Links software [8,17].

2.2. Glycosidase digestion

Purified fibrinogen (50) μg was taken up in 50 μl of 50 mM Tris/HCl pH 7.5 containing 10 mM EDTA, 1% NP-40 and incubated (16 h, at 30 °C) with 5 U of N-glycosidase F (Roche). One microlitre was analysed by reducing SDS–PAGE to confirm cleavage of the oligosaccharide and the remainder reduced, as above, and subjected to reverse phase HPLC to isolate the Bβ and γ chains for mass analysis.

For the removal of galactose from non-reducing termini, 50 μg of fibrinogen was taken up in 50 μl 50 mM phosphate buffer pH 7, 1 mM Mg Cl_2 1.5 mM dithiothreitol and 1 μg of *Streptococcus pneumoniae* β1,4 galactosidase (Calbiochem) added. Digests were incubated overnight at 37 °C, and the fibrinogen precipitated with ammonium sulphate prior to reduction, reverse phase HPLC and mass spectrometry. Of note, neither the Roche or the Sigma β1,4 galactosidase from *Escherichia coli* were capable of cleaving the terminal galactose from fibrinogen. Although all three enzymes rapidly cleaved the reporter chromogenic substrate 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal), only the *Streptococcus pneumoniae* enzyme was able to cleave the terminal Gal residues from fibrinogen.

2.3. Isolation of glycopeptides

Ten micrograms of Fibrinogen was boiled (5 min) in 1 ml 50 mM NH_4HCO_3, 0.2 mg trypsin added, and the digest incubated for 24 h at 37 °C. After centrifugation, the supernatant was applied to a 1 ml HiTrap Conconavilin A Sepharose 4B column (GE Healthcare) equilibrated in 0.1 M NH_4HCO_3, pH 6.7, 1 mM $MnCl_2$ and 1 mM $CaCl_2$.The column was washed with 0.1 M NH_4HCO_3 pH 6.7, and the unbound fraction was collected. After further washing with 5-column volumes, the bound fraction was eluted with 0.1 M α methyl glucoside. Bound and unbound fractions were dried and taken up in 0.5 ml 0.1% HCOOH. Fractions (10 μl) were then diluted into 50% acetonitrile 0.1% HCOOH and analysed by direct injection into the electrospray source.

3. Results

3.1. Patient

The principal patient examined here was a 58-year-old heavy smoker diagnosed as having an abscess on his lung. He had been ill for 2 weeks prior to our "day 1" plasma sample, at which time he had a CrP of 298 mg/L. Plasma samples were obtained daily over the subsequent 8 days after which time his CrP had fallen to 160 mg/L. Fibrinogen was purified from each of the plasmas, and the individual chains isolated by reverse phase HPLC prior to analysis by electrospray ionisation mass spectrometry (ESI MS).

3.2. Fibrinogen Bβ and γ chain analysis

Examination of γ chains from normal and quiescent state control plasma with a CrP of <5 mg/L showed the expected major monosialo isoform at 48,375 Da together with its disialo isoform (+291) at 48,668 Da [5,8,18] (Fig. 1). Patient chains showed a very different pattern with peaks at 48,216, 48,375 and 48,666 Da. The dominant 48,216 species was 159 Da less than the dominant control isoform possibly reflecting loss of hexose (−162 Da) from the oligosaccharide, because if a truncation of the protein was responsible for the mass decrease, then the disialo isoform at +291 Da would also have a similar (−162 Da) companion peak. Kinetically, the amount of what we have termed des-Gal γ decreased progressively from 55% to 39% during the acute phase recovery from a CrP of 298 to 160 mg/L, and the amount of disialo γ increased from 8% to 16% of the total over the 8 days of recovery.

Analysis of Bβ chains also showed the familiar mono and disialo isoforms (+291 Da) at 54,198 and 54, 499 Da [5,8,17,18]. Patient Bβ chains, on the other hand, were composed of additional lower mass peaks at −160 Da and a further component at 199 Da lower again. These were surmised to represent des-Gal and desNAcGlc-Gal, which would have successive mass decreases of 162 and 203 Da, respectively. The amount of Des-Gal β decreased from 35% to 20% and desNAcGlc-Gal from 12% to 5%, while disialo Bβ chains increased from 12% to 20 % over the 8 days of sampling.

The measured mass decrease of the γ chain (−159 Da) could correspond to loss of its N-terminal Tyr (−163 Da) or to loss of a terminal galactose residue (162 Da) from its asialo oligosaccharide fork. Tryptic peptide maps of patient γ chains were however very similar to controls; specifically, there were no differences in intensities of ions derived from either the N- or C-terminal peptides (not shown).

3.3. Digestion with N-glycosidase F

To confirm the carbohydrate moiety as the source of the acute phase variation, fibrinogen was incubated with N-glycosidase F, which cleaves the glycosidic bond between Asn and NAcGlc, and the individual chains isolated by HPLC. Theoretically, this should involve decreases of 1,915 and 2,206 Da for the mono and disialo isoforms, respectively, and the production of a single unadorned protein product. This is exactly what was observed in the control γ chains, where mass decreases of 1,919 and 2,202 Da yielded a polypeptide of mass 46,474 Da (Fig. 2). The three different components of the patient γ chains were similarly normalised down to a single naked polypeptide of 46,472 Da,

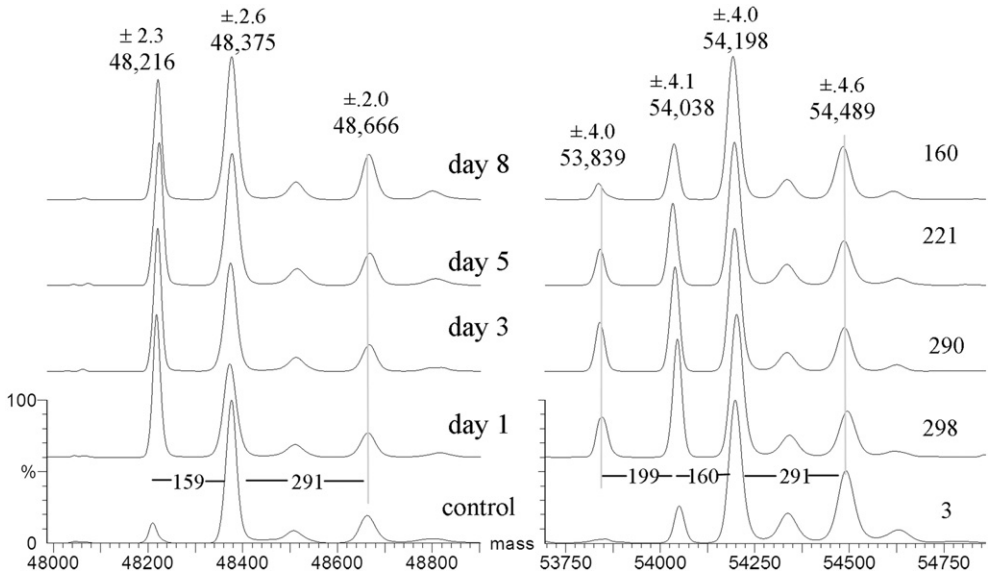

Fig. 1. Transformed ESI spectra of isolated γ (left panel) and Bβ chains (right panel) showing normal control (bottom pane) and selected chains from days 1 to 8 of the acute phase response. The displayed masses are the averages of the eight measurements with standard errors. Mass differences from the base peak (the monosialo chain) are also shown together with CrP values in mg/mL (far right) for the various days. Spectra of patient γ chains showed the major isoform had a mass decrease of 159 Da, while patient Bβ chains also showed a shift to lower masses with novel signals at −160 and −199 Da, most probably corresponding to progressive loss of Gal (162 Da) and NAcGlc (203 Da) from the asilo branch of the oligosaccharide.

confirming that the heterogeneity of the protein was contained within its oligosaccharide side chain. Examination of Bβ chains from the control also showed the mono and disialo isoforms had decreased by 1,921 and 2,217 Da to give a 52,284 polypeptide after N-glycosidase F treatment. Similarly, the four isoforms (desNAcGlc-Gal, des-Gal, monosialo and disialo) of the patient Bβ chains were normalised to a single 53,289 Da protein (Fig. 2).

3.4. Digestion with β1-4 galactosidase

In order to establish the precise cause of the Bβ and γ chain variation, patient and control fibrinogen were incubated with β1-4 galactosidase from *Streptococcus pneumoniae*. This exoglycosidase hydrolyses non-reducing terminal β1-4 linked galactose residues. As only the monosialo isoform has such a terminal, the minor disialo isoform should remain unaffected by galactosidase, but both the control and the patient chains should normalise to the patient pattern. This is precisely what was observed. Control γ chains decreased from 48,390 to 48, 219 Da to match the aberrant 48,216 Da γ chain of the patient, while only the 48,370 Da chain of the patient was hydrolysed (Fig. 3). Similar results were obtained for the Bβ chain with the monosialo control chain decreasing to 54,031 Da and matching the patient chains at 54,035 Da (not shown).

3.5. Glycopeptide isolation and peptide analysis

To confirm the absence of galactose as the cause of Bβ and γ chain acute phase heterogeneity, tryptic glycopeptides were isolated by

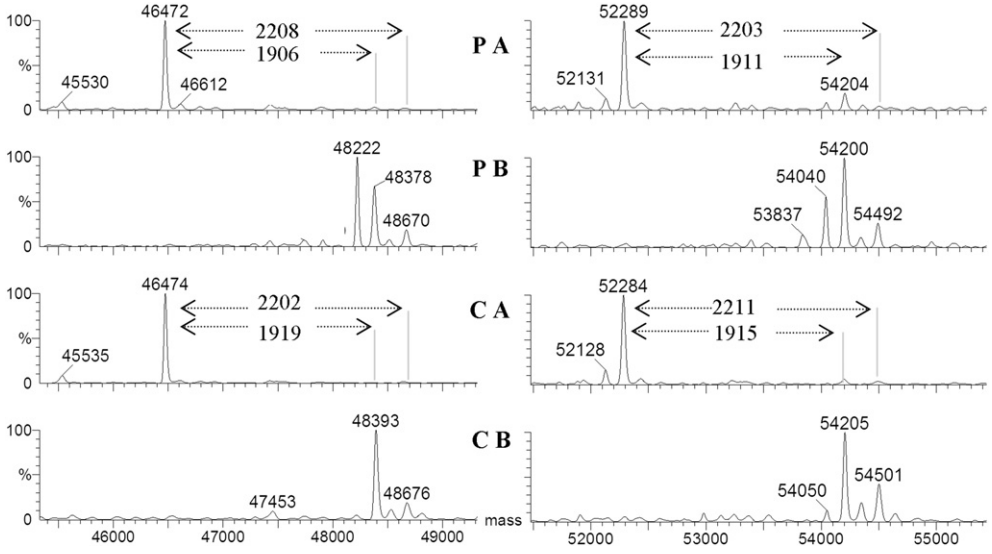

Fig. 2. Transformed ESI spectra of γ (left panel) and Bβ chains (right panel) isolated from fibrinogen before and after incubation with N-glycosidase F. Control before (CB) and control after (CA) incubation, and day 2 patient fibrinogen before (PB) and after (PA) incubation. Measured mass differences are indicated in comparison to the expected shifts of 1,919 and 2,206 Da. The normalisation of patient chains on incubation with the endoglycosidase establishes the carbohydrate moieties as the source of the acute phase mass variation.

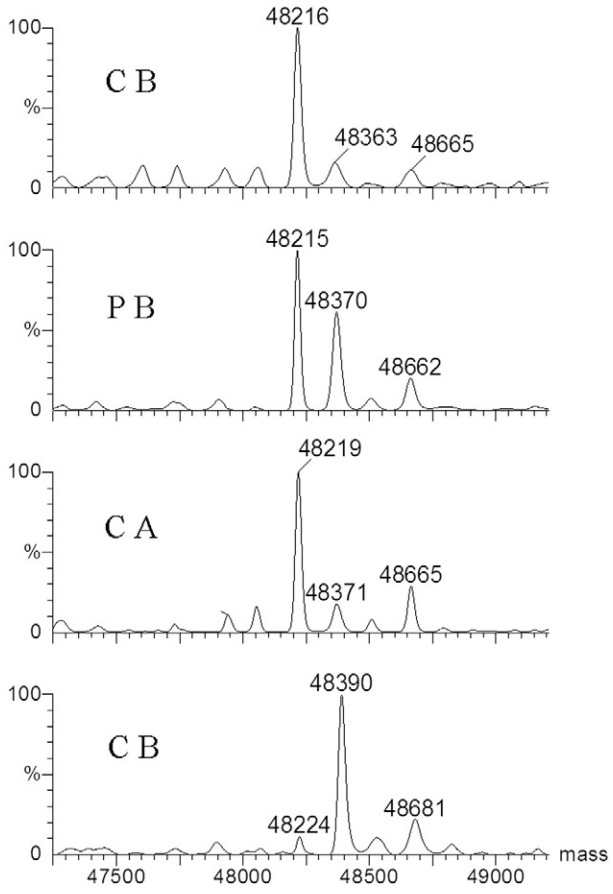

Fig. 3. Transformed ESI spectra of γ chains isolated from fibrinogen before and after incubation with *Streptococcus pneumoniae* β1,4-galactosidase. Control before (CB) and control after (CA) incubation; patient before (PB) and patient after (PA) incubation. Only the monosialo isoform is susceptible to hydrolysis as the 48,216 and 48,662 Da species lack terminal galactose residues.

affinity chromatography on concanavalin-A Sepharose after elution with α methylglucoside. ESI m/z maps confirmed that both oligosaccharide chains were exclusively of the biantennary type. Doubly and triply charged ions of β41 and γ5, the expected glycopeptides, were detected together with companion species lacking one sialic acid residue (Fig. 4A). In addition, a γ4-5 peptide was also detected; its presence being the result of the negative impact of the flanking aspartic acid residues on cleavage at Lys38. Na^+ and K^+ ions of γ5 (1239 and 1245 m/z) were detected along with the fully protonated 1232 ion and similar triplet ions were seen for $\beta41^{+3}$ at 1270, 1277 and 1282 m/z. The structure of the γ4-5 peptide that gives rise to the 1346 and 2019 ions is shown in Fig. 4 panel C. If as, surmised, this peptide lost galactose (162 Da), then companion ions would be expected at 1292 and 1938 m/z. Surprisingly, these ions were not present, and similarly there was no evidence of loss of galactose from β41 in this concanavalin-A purified peptide fraction.

Initially, this seemed to negate the finding that loss of galactose was the underlying cause of the acute phase variation, and to resolve this paradox, we examined total digests of individually isolated fibrinogen chains that had no prior lectin affinity isolation (Fig. 4B). Here the same pattern of γ ions was seen together with expected non-glycopeptides ions such as 2161 (γ8), 1418 (γ32), 1547 (γ42) or 1895 (γ36). More importantly, the predicted new glycopeptide ions were seen at 1292, 1938 and 1767 m/z corresponding to γ4-5 and γ5 peptides lacking galactose. Notably, these were of similar intensity to their full-length companion peptides (1346, 2019, 1848 respectively), indicating that day 7 plasma contained 50% des-Gal γ chains. This was in excellent agreement with estimates of 46% from analysis of the intact fibrinogen chains from day 7 plasma (Fig. 1).

From our previous mass analysis of intact γ chains from over 200 individuals with hypo- or dysfibrinogenemia (and assumed normal CrP), we have rarely see peaks of over ~5% in the des-Gal γ position at −162 Da. In an attempt to confirm the loss of galactose related to a sustained acute phase response, we examined an additional nine plasma samples from another individual with high CrP values. The patient in this case was a 57-year-old alcoholic with a sustained

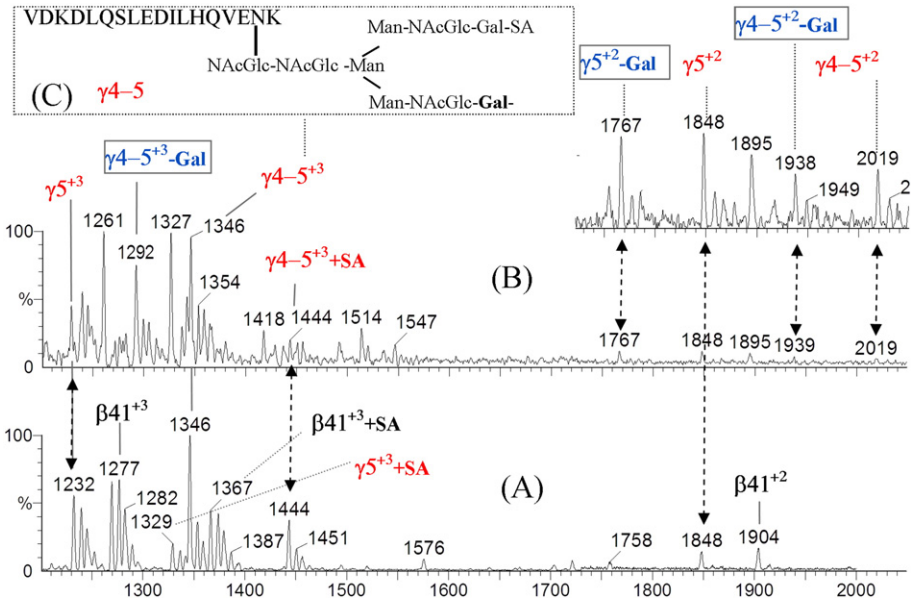

Fig. 4. ESI tryptic peptide maps. (A) Conconavalin-A Sepharose bound glycopeptides from digests of total fibrinogen from patient plasma. The M + 3 and M + 2 ions of β41 together with its disialo isoform (+SA) are indicated; Na^+ and K^+ adducts are seen in addition to the protonated ion (H^+) at 1277, 1280 and 1270, respectively. The γ chain glycopeptide was detected as γ5 and γ4-5, with (+SA) or without one sialic acid. (B) Tryptic digest of isolated fibrinogen γ chains from day 4 patient plasma; the insert shows repeat high mass scan of same digest, glycopeptides derived ions are labelled. The principal species detected were M + 3H and M + 2H ions of γ5 and γ4-5. Most significantly, there were companion ions to $\gamma4\text{-}5^{+3}$(1346) and $\gamma4\text{-}5^{+2}$(2019) at 1292 and 1938 m/z, respectively, that reflect the loss of galactose (162 Da). Similarly, the loss of ~50% of the galactose from $\gamma5^{+2}$ is evidenced by the similar amplitudes of the 1767 and 1848 ions. (C) Structure of the principal γ4-5 biantennary glycopeptide that terminates in sialic acid and galactose; instead, the variant peptide terminates at the SA and NAcGlc.

illness and suffering from pneumonia. His CrP values ranged from 415 to 190 mg/L over a 10-day period. Over the first 8 days, his des-Gal γ fibrinogen progressively rose from 15% to 42% of the total and then decreased to 34% by day 10.

4. Discussion

Here we have shown that, unlike α1 antitrypsin [10], the oligosaccharide side chains of fibrinogen remain exclusively of the biantennary type during an acute phase response. Instead of the increased branching seen in antitrypsin, post-translational variation of fibrinogen involves decreases in the length of one of the γ and Bβ chain forks (Fig. 4C). Mass analysis of isolated intact chains suggested loss of galactose was the underlying cause of the −162 Da mass variations, and this was supported by digestion with N-glycosidase F, which normalised the acute phase and quiescent fibrinogen to the same mass of 46,474 for the γ and 52,289 Da for the Bβ chain (Fig. 2). Incubations with *Streptococcus pneumoniae* β1,4-galactosidase further confirmed that the absence of galactose underpinned the structural difference between the quiescent and the inflammatory state (Fig. 3).

Despite these clear findings, maps of glycopeptides isolated by affinity chromatography on conconavalin-A Sepharose failed to show any glycopeptides that lacked galactose. On the other hand, maps of non-lectin-fractionated γ chain peptides showed equal portions of the γ5 (and γ4-5) peptide with and without their terminal galactose. The conclusion from this can only be that the modification itself decreased conconavalin-A affinity and that the des-Gal peptides eluted in the unbound, or buffer-wash fractions. Examination of the unbound fraction (not shown) showed no glycopeptides, suggesting the des-Gal peptides eluted in the discarded low stringency washes. The principal requirement for lectin binding would seem to be the presence of *cis*-diols on the sugar residues, and these are provided by mannose and galactose residues, which have *cis*-hydroxyl groups in the 2′3′ and 3′4′ positions, respectively. Loss of one galactose residue should therefore reduce, but probably not prevent lectin binding, resulting in exclusion of des-Gal peptides from either the bound or unbound fraction.

In the sustained inflammatory response examined here, des-Gal γ fibrinogen became the dominant γ chain species present exceeding 55% of the total (Fig. 1). Moreover, in the case of the Bβ chain, there was additional fraying of the carbohydrate structure because in addition to des-Gal β, which accounted for 35%, substantial amounts (12%) of further truncated des-NAcGlc-Gal was also present.

Alterations in the oligosaccharide configuration of plasma glycoproteins can have a profound impact on haemostasis [16]. As an example the physiological variant, antithrombin β, which lacks its Asn135 oligosaccharide, has a higher heparin affinity than antithrombin α [19]. So although the β isoform makes up only 5–10% of the total, it is actually the major physiological regulator of thrombin activity [20]. Small changes in carbohydrate structures can also have a significant impact on circulatory clearance. For example (in rats), the half-life of von Willebrand factor decreases from 240 to 5 min after incubation with neuraminidase, which unmasks the underlying Gal residues and triggers uptake of the protein by the asialoglycoprotein receptor [21]. Also, it has been shown that the inactivation of the sialyltransferase that adds CMP-activated acetylneuraminic acid into penultimate Gal residues (ST3 Gal-IV) significantly increases von Willebrand Factor uptake by this receptor [21,22].

What impact might the loss Gal have on fibrinogen? First, it is well established that a decrease in the sialic acid content lessens the electrostatic repulsion between fibrin monomers during polymerisation [6,9,23]. Hence, acute phase fibrinogen would have a shorter clotting time because if there is no galactose terminal available, then the outer sialic acid cannot be attached by CMP-NAcNeu Gal transferase. As it turns out, this is a desirable outcome because part of the acute phase response is to protect against the threat of haemorrhage.

The second predictable impact on fibrinogen would be an extended half-life and ramped up plasma concentration because fibrinogen is normally removed from circulation not only by fibrinolytic consumption but also by the hepatic asialoglycoprotein receptor. What this receptor actually recognises, however, is two exposed Gal residues, and clearly if there is no Gal on one branch, then the receptor can never “see” two residues. This is not to say the half-life will become infinite because in the 340 kDa fibrinogen molecule, there are a total of four biantennary oligosaccharides any one of which can reveal two Gal residues and provoke turned over. In support of this, we have analysed many hundreds of fibrinogen samples by ESI MS and never observed a single incidence of a circulatory fibrinogen molecule lacking both sialic residue.

5. Conclusion

In conclusion, in an acute phase, the carbohydrate structure of fibrinogen becomes modified in such a way as to increase the proteins concentration and the rate of fibrin polymerisation and clot formation, helping to maintain haemostasis.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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