Pregnancy associated changes in the glycosylation of Tamm-Horsfall glycoprotein.

EXPRESSION OF SIALYL LEWIS\(^x\) SEQUENCES ON CORE 2 TYPE O-GLYCANS DERIVED FROM UROMODULIN*

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Pregnancy dependent O-glycosylation
ABSTRACT

Tamm-Horsfall glycoprotein (THP) is a major glycoprotein associated with human urine that binds pro-inflammatory cytokines and also inhibits in vitro T cell proliferation induced by specific antigens. THP derived from human pregnancy urine (designated uromodulin) has previously been shown to be thirteen fold more effective as an inhibitor of antigen-induced T cell proliferation than THP obtained from other sources. Structural analysis of human THP and uromodulin has for the first time revealed that these glycoproteins are O-glycosylated. THP from nonpregnant females and males expresses primarily core 1 type O-glycans terminated with either sialic acid or fucose but not the sialyl Lewis^x epitope. By contrast, the O-glycans linked to uromodulin include unusual core 2 type glycans terminated with one, two or three sialyl Lewis^x sequences. The specific association of these unusual carbohydrate sequences with uromodulin could explain its enhanced immunomodulatory effects compared to THP obtained from males and nonpregnant females. Analysis of THP from one of the pregnant females two months postpartum showed a reversion of the O-glycan profile to that found for a non-pregnant female. These data suggest that the glycosylation state of uromodulin could be under the regulation of steroidal hormones produced during pregnancy. The significant physiological implications of these observations are discussed.
INTRODUCTION

Tamm-Horsfall glycoprotein (THP) is the most abundant glycoprotein in human urine (1,2). The precise physiological role of THP remains enigmatic, despite considerable investigation of this glycoprotein over the past fifty years. THP was shown to block hemagglutination induced by influenza, mumps and Newcastle disease viruses (1,2). THP has been postulated to be essential for protecting the kidneys from bacterial infections (3,4). THP has also been implicated as a potential component necessary for maintenance of the electrolyte balance in the nephron (5). In addition, THP may also play a significant role in several pathological conditions involving the kidney, including acute renal failure, urinary tract infection, stone formation and interstitial nephritis (6).

In 1973, two studies suggested that human chorionic gonadotropin (hCG) isolated from pregnancy urine suppressed specific immune activities in vitro (7,8). However, this immunosuppressive activity turned out to be due to a contaminant of the hCG preparation (9,10). Muchmore and Decker later used lectin affinity chromatography, gel separation, and isoelectric focusing to purify a glycoprotein from human pregnancy urine that mediated this immunosuppressive activity (11). They designated this glycoprotein "uromodulin" to account for its origin and its immunomodulatory activities. Amino acid sequence analysis (12,13) revealed that uromodulin represented THP glycoforms produced during pregnancy.

Uromodulin was initially shown to inhibit antigen-induced T cell proliferation at relatively low concentrations (11). Subsequent investigations by Muchmore and colleagues indicated that uromodulin could also bind to recombinant interleukin-1 and interleukin-2 (rIL-1 and rIL-2) and recombinant tumor
necrosis factor (rTNF) via its oligosaccharide sequences (14-16). The glycans associated with uromodulin have been proposed to be essential for its immunosuppressive and cytokine binding activities (12,14-16). By contrast, another study indicated that both THP and uromodulin would bind to IL-1 and TNF, but only after these cytokines were either denatured or immobilized on solid surfaces (17). However, a more recent study indicates that oligosaccharides associated with THP block IL-1 binding to immune effector cells and to an IL-1 dependent cell line (18). The investigators in this study also provided strong evidence that N-linked glycans terminated with GalNAc and a sulfate group mediate these immunomodulatory activities in vitro (18). This demonstration that THP or its derivative oligosaccharides can also mediate cytokine binding and specific responses in immune cells suggests that this glycoprotein is functionally equivalent to uromodulin. However, Muchmore and coworkers suggested that uromodulin was a 13-fold more active inhibitor of antigen-specific T cell proliferation than THP (12). The observation that THP and uromodulin express identical polypeptide sequences suggests that pregnancy related changes in post-translational modifications could be responsible for their differential immunosuppressive activities.

The present investigation was undertaken to determine if THP and uromodulin are differentially glycosylated. Several pregnancy associated changes in the glycosylation of THP were found, the most notable being that uromodulin specifically expresses unusual core 2 type O-linked glycans terminated with up to three sialyl Lewis sequences.
EXPERIMENTAL PROCEDURES

Purification of THP/uromodulin -- THP or uromodulin was purified from urine from a range of individuals. Each sample was prepared individually. THP was isolated using the same procedure originally employed by Tamm and Horsfall (1). The urine sample was adjusted to pH 7.0 using sodium hydroxide. Sodium chloride was then added and dissolved to give a final concentration of 0.58M NaCl. The sample was then left at 4°C for 5 hours followed by centrifugation at 1000 rpm for 30 minutes. The supernatant was removed and the precipitate resuspended in 2 ml of 0.58 M sodium chloride. The sample was then centrifuged at 4000 rpm for 15 minutes. The supernatant was again removed and the precipitate resuspended in 2 ml of water. This purified THP/uromodulin was then transferred into a regenerated cellulose dialysis membrane with 12-14 kDa nominal molecular weight cutoff (Cellu-Sep) and dialysed against 4 x 2.5 l of water at 4°C, the water being changed at 12 hour intervals for 2 days. After dialysis the THP/uromodulin was lyophilized.

Reduction and carboxymethylation -- THP/uromodulin was subject to reduction and carboxymethylation of S-S bridged cysteine residues as previously described (19) with three modifications. Firstly, the reduction was performed under a nitrogen atmosphere; secondly, the iodoacetic acid incubation was performed under a nitrogen atmosphere at 37°C; and thirdly the reaction was terminated and desalted by dialysis against 4 x 2.5 l of 50mM ammonium bicarbonate pH 8.5 at 4°C for 2 days, the dialysate being changed every 12 hours. After dialysis the sample was lyophilized.
Tryptic digestion--THP/uroomodulin was digested with trypsin using a 1:100 wt:wt ratio of trypsin (EC 3.4.21.4, Sigma) to THP/uroomodulin as described (19). The incubation period for the digestion was 5 hours. Following termination of the digestion the sample was lyophilized.

PNGase F digestion--THP/uroomodulin was digested with 3 units of PNGase F (EC 3.5.1.52, Roche Molecular Biochemicals) as described (19). The reaction was terminated by lyophilization and the released N-glycans separated from peptides and O-glycopeptides by Sep-Pak as described (19). Prior to loading onto the conditioned Sep-Pak the sample was dissolved in 200 µl of 5% acetic acid.

Digestion with Vibrio cholerae neuraminidase--Tryptic O-glycopeptides from uromodulin, present in the 20% 1-propanol Sep-Pak fraction, were dissolved in 50mM ammonium acetate pH 4.5 and digested with 25 milliunits of Vibrio cholerae neuraminidase (EC 3.2.1.18, Roche Molecular Biochemicals) for 24 hours at 37°C. Following digestion the sample was lyophilized.

Reductive elimination--THP/uroomodulin was subject to reductive elimination as described (19). Following termination of the reaction using glacial acetic acid, the 20% and 40% 1-propanol fractions were combined prior to Dowex chromatography.

Chemical defucosylation--The released O-glycans were dissolved in 50 µl of hydrofluoric acid (Sigma) and placed on ice for 30 minutes. The reaction was terminated by drying under a stream of nitrogen.

Periodate cleavage--A solution of 2mM sodium periodate in 100mM ammonium acetate pH 6.5 was prepared, wrapped in foil and placed in the fridge for one hour. Released, dried O-glycans were then dissolved in 50 µl of this reagent, wrapped in foil and placed on ice in the fridge overnight. The reaction was terminated by the addition of 2 µl of ethylene glycol and lyophilized. The products of periodate
cleavage were reduced using 200 µl of a 10mg/ml solution of sodium borohydride in 2M ammonium hydroxide. The reaction was allowed to proceed at room temperature for 2 hours. The reaction was terminated by addition of glacial acetic acid until all the sodium borohydride had been neutralized. The sample was subject to Dowex chromatography, borate removal, permethylation and Sep-Pak clean-up using an acetonitrile gradient as described (19).

*Chemical derivatization for FAB-MS and GC-MS analysis*--Released glycans were permethylated using the sodium hydroxide procedure and purified by Sep-Pak using an acetonitrile gradient as described (19). Partially methylated alditol acetates were prepared from the permethylated glycans as described (20) and analysed by GC-MS.

*Mass spectrometric analyses*--Fast atom bombardment mass spectrometric analysis of permethylated glycans was performed using a ZAB 2SE 2FPD double focusing mass spectrometer fitted with a cesium ion gun operating at 30 kV. The matrix used was monothioglycerol and all samples were dissolved in methanol prior to loading. Data was collected and analysed using VG Opus® software. Linkage analysis of partially methylated alditol acetates was carried out using an MD 800 gas chromatography mass spectrometer. Chromatographic separation was achieved using a 30m x 0.25mm i.d. RTX-5 fused silica capillary column (Restek Corp.). The sample was dissolved in hexanes and injected onto the column at 65°C. The column was held at this temperature for one minute and then increased at a rate of 8°C/min to 290°C.
RESULTS

Structural analysis strategy

THP or uromodulin were isolated as described in Experimental Procedures from urine samples of three males, three non-pregnant females and two pregnant females. Additionally THP was isolated from one of the pregnant females two months postpartum. All samples exhibited a single band on SDS-PAGE at about 90kDa (data not shown). Peptide mapping by fast atom bombardment mass spectrometry (FAB-MS) confirmed the presence of the THP polypeptide (data not shown). Putative O-glycans were released by reductive elimination, permethylated, and purified on a Sep-Pak cartridge which was eluted with a stepwise gradient of aqueous acetonitrile. The permethylated glycans, which were recovered in the 35% and 50% acetonitrile fractions, were analyzed by FAB-MS and linkage analysis. Under the conditions used for reductive elimination a portion of the N-glycans is also released. Their presence was taken into account in the interpretation of the FAB and linkage data.

Analysis of glycans released from male THP by reductive elimination

FAB-MS analyses of the 35% acetonitrile fractions from male THP showed several low molecular weight O-glycans, the most abundant of which (m/z 895 and 1256) were present in all three samples (Fig. 1, Table 1). These masses correspond to compositions NeuAcHexHexNAcitol and NeuAc$_2$HexHexNAcitol respectively, and are consistent with sialylated and disialylated T-antigen structures (NeuAc$\alpha$2-3Gal$\beta$1-3GalNAc and NeuAc$\alpha$2-3Gal$\beta$1-3(NeuAc$\alpha$2-6)GalNAc). These simple core 1 type glycans are found in many glycoproteins including glycophorin, erythropoietin and plasminogen. There is some variation between individuals with respect to the relative abundance of the above two glycans and the compositions
of minor structures present (Table 1). For example m/z 1140 (NeuAcHexHexNAcHexNAcitol) was detected in only two samples. This glycan probably carries the Sd\(^a\) antigen (GalNAc\(\beta\)_1-4(NeuAc\(\alpha\)_2-3)Gal\(\beta\)_1-) which is known to be present on the antennae of THP N-glycans (21). The 50% acetonitrile fractions showed molecular ions attributable to high mannose N-glycans (Fig 1b, Table 1). These are readily identifiable because of their unique compositions and because they exhibit pairs of molecular ions separated by 16 mass units due to the incomplete reduction which is characteristic of N-glycans released under reductive elimination conditions. O-glycans, in contrast, are fully reduced under the conditions employed in our experiments. No significant signals corresponding to O-glycans were observed in the 50% fraction.

*Analysis of glycans released from female THP by reductive elimination*

Figure 2 and Table 1 show FAB data obtained from the analysis of THP from a single female. Data from a further two females are also shown in Table 1. Comparison with the male data allows the following conclusions to be drawn: (i) The two major glycans observed in the male (NeuAc\(_1\) and \(_2\)HexHexNAcitol) are also found in female THP but their abundance varies significantly between individuals and is generally lower in females than males; (ii) Like the male, the non-pregnant female appears to contain relatively simple O-glycans containing no more than six sugar residues; (iii) In two of the samples analysed, an abundant signal at m/z 1157 was detected corresponding to FucHex\(_2\)HexNAcHexNAcitol. One of these two samples also exhibited a minor signal corresponding to the presence of a second fucose residue (m/z 1331). These glycans probably contain Lewis\(^x\)\(^a\) structures (Gal\(\beta\)_1-4(Fuc\(\alpha\)_1-3/4)GlcNAc\(\beta\)_1), evidence for which is provided by a fragment ion at m/z 638 (A-type fragment ion of composition FucHexHexNAc\(^+\)).
Uromodulin contains O-glycans not present in THP

Figure 3a and Table 2 show FAB data obtained from the analysis of the 50% Sep-Pak fraction of permethylated glycans from uromodulin obtained from a single pregnant female. Data from a second pregnant female are also shown in Table 2. The data show striking differences when compared with THP. The most notable feature is the presence of major molecular ions corresponding to large O-glycans rich in fucose and sialic acid which are completely absent in the THP samples. The most abundant of these components are observed at m/z 1519 (NeuAcFucHex$_3$HexNAcHexNAcitol), m/z 1880 (NeuAc$_2$FucHex$_2$HexNAcHexNAcitol) and m/z 2504 (NeuAc$_2$Fuc$_2$Hex$_3$HexNAc$_2$HexNAcitol). Weaker signals are present at higher mass that correspond to larger O-glycans carrying additional fucosyl and sialyl residues up to a composition of NeuAc$_3$Fuc$_3$Hex$_4$HexNAc$_3$HexNAcitol (m/z 3487). The abundant signal at m/z 999 (NeuAcHexHexNAcFuc$^+$) is consistent with the presence of either sialyl Lewis$^a$ or sialyl Lewis$^b$. The majority of the putative Lewis epitopes are sialylated since a signal at m/z 638 (not shown), representing FucHexHexNAc$^+$, is minor compared to m/z 999. No signals were detected for fragment ions corresponding to lactosamine repeats, indicating that the higher mass structures are most likely produced by branching of O-glycans rather than chain elongation.

Modification of O-glycan profile following parturition

Analysis of THP/uromodulin isolated from the first pregnant female two months after birth of a male baby showed a dramatic change in the O-glycan profile (Fig 3b). Notably the sialylated and fucosylated O-glycans observed in uromodulin had virtually disappeared (for example, compare m/z 1519, 1880 and 2504...
in Figs 3a and 3b) whilst the profile of small O-glycans which eluted in the 35% Sep-Pak fraction was largely unaffected and was similar to the non-pregnant state (Figs. 4a and 4b).

*Confirmation of the presence of fucose in uromodulin O-glycans*

Reductively eliminated glycans from uromodulin were subjected to hydrofluoric acid defucosylation and the products of the reaction were permethylated and analysed by FAB-MS after Sep-Pak purification. The data show that the signals attributed to fucose containing O-glycans (Table 2) have either disappeared or are considerably reduced in intensity (compare Figs 5a and b). New signals are present (Fig 5b) consistent with loss of fucose from the original structures such as m/z 1344 (loss of fucose from m/z 1519) and m/z 1706 (loss of fucose from m/z 1880). The fragment ion originally present at m/z 999, representing a possible sialyl Lewis epitope, has also virtually disappeared. The signal at m/z 825 (NeuAcHexHexNAc+ fragment ion) is more intense than was originally detected. This product is expected following the defucosylation of the putative sialyl Lewis epitope.

*Desialylation of uromodulin O-glycans*

Tryptic glycopeptides were desialylated using *V. cholerae* sialidase and glycans were released by reductive elimination, permethylated and analysed by FAB-MS. The data showed the loss of signals with compositions consistent with the presence of sialic acid (compare Figs 6a and b). New signals were observed at the expected desialylated masses. Thus loss of one and two sialic acids respectively from m/z 1519 and 1880 yields m/z 1157 whilst similar losses from m/z 2142 and 2504 yields m/z 1781. The new signals at m/z 2230 and m/z 2404 represent desialylation of the higher mass structures shown in Fig.6a, specifically m/z 2230 is derived from m/z 2952 and m/z 3313, whilst m/z 2404 is derived from m/z 3126.
and m/z 3487. The sialyl Lewis\textsuperscript{\As} fragment ion (m/z 999) disappeared concomitant with a significant increase in the signal at m/z 638 (FucHexHexNAc\textsuperscript{+}), consistent with the loss of sialic acid from this epitope.

**Periodate cleavage defines core types and antennae backbones of uromodulin O-glycans**

Mild periodate oxidation cleaves linear sections of sugar structures if vicinal hydroxyl groups are present. Thus, with respect to the O-glycans of uromodulin, the GalNAcitol and any sialic acids are sensitive to this reagent. Reductively eliminated glycans were subjected to mild periodate oxidation followed by reduction, permethylation, Sep-Pak clean-up and FAB-MS analysis. The FAB data for the 35% and 50% Sep-Pak fractions (Fig. 7a and b respectively, Table 3) showed molecular ions consistent with periodate cleavage of the GalNAcitol residue producing two distinct sets of products, one containing the C1 to C4 carbons of the GalNAcitol (denoted C4) attached to the 3-linked antenna(e), and the other containing the C5 and C6 carbons (denoted C2) attached to the 6-linked antenna(e). Thus, m/z 719, 1069, 1342, 1587, 1791, 1967, 2065 and 2240 are attributable to 3-linked antennae because their masses are consistent with the presence of a C4 moiety at the reducing end (Table 3). Similarly, m/z 562, 736, 835 and 1009 are consistent with 6-linked antennae (attached to the C2 moiety). From these data it is evident that the sialyl Lewis\textsuperscript{\As} epitope is present on both 3-linked (m/z 1342) and 6-linked (m/z 1009) antennae, although the significantly larger signal at m/z 1009 compared to m/z 1342 indicates that attachment to carbon 6 is more common in these smaller structures. The higher mass signals corresponding to 3-linked antennae have compositions consistent with the possible presence of lactosamine repeats. However, the absence of A-type fragment ions characteristic of polylactosamine sequences in the spectra of the intact glycans (see, for example Fig
3a), suggests that polylactosamine structures are unlikely to be present. Consequently 3-linked antennae of compositions NeuAcFuc\textsubscript{2}Hex\textsubscript{3}HexNAc\textsubscript{2}-C\textsubscript{4}, NeuAc\textsubscript{2}FucHex\textsubscript{3}HexNAc\textsubscript{2}-C\textsubscript{4} and NeuAc\textsubscript{2}Fuc\textsubscript{2}Hex\textsubscript{3}HexNAc\textsubscript{2}-C\textsubscript{4} are likely to be branched. Their compositions are consistent with the presence of two sialyl Lewis\textsuperscript{x} epitopes.

*Linkage analysis of reductively eliminated uromodulin glycans*

Linkage analysis data for uromodulin are shown in Table 4. Notable features of the linkage data are: (i) 3,6-GalNAcitol is significantly more abundant than 3-GalNAcitol, which is consistent with core 2 type structures being dominant in pregnant samples, and is significantly reduced postpartum; (ii) the presence of 3,6 Gal supports the periodate data which provided evidence for the existence of branched structures; this component was very minor post-pregnancy in accord with the FAB data showing loss of the high molecular weight O-glycans; (iii) the absence of detectable levels of 6-Gal indicates that the sialic acids are attached at the 3-position of galactose; this was confirmed by linkage analysis performed after *V. cholerae* desialylation which showed a loss of 3-linked galactose and an increase of terminal galactose; (iv) the presence of 3,4-GlcNAc, which disappears after HF defucosylation without the concomitant appearance of 3-linked GlcNAc, confirms that sialyl Lewis\textsuperscript{x} and not sialyl Lewis\textsuperscript{a} is present in the O-glycans; (v) the terminal GalNAc is supportive of the possible presence of the Sd\textsuperscript{a} antigen in a minority of glycans; (vi) the variously linked mannoses are derived from the “contaminating” N-glycans.

*Structures of the high molecular weight O-glycans in uromodulin*

Taking into consideration the FAB-MS, linkage, exoglycosidase, periodate and defucosylation data, we arrive at the following structural conclusions: (i) uromodulin carries a range of O-glycans which are not
present in THP; (ii) these glycans are rich in sialyl Lewis^x and the majority have the core 2 type (Galβ1-3(GlcNAcβ1-6)GalNAc), in contrast with THP which is mostly core 1 type; (iii) the periodate data suggest that the 6-linked antenna has four possible sequences \textit{viz} Gal-GlcNAc, NeuAc-Gal-GlcNAc, Lewis^x and sialyl Lewis^x; in contrast the 3-linked antennae show significantly greater heterogeneity with at least eight structures being observed; (iv) the largest glycan observed is a tetradecamer whose composition (NeuAc,Fuc,Hex,HexNAc) is consistent with the presence of three sialyl Lewis^x moieties. Structures taking account of these conclusions are summarised in Fig 8.
DISCUSSION

The primary goal of the present study was to determine if any major changes in the glycosylation of THP occurs during human pregnancy. To carry out this investigation, uromodulin was isolated from human pregnancy urine by the salt precipitation method previously employed to isolate THP from non-pregnant females and males (2). This consistent approach to the isolation of THP and uromodulin is important, because uromodulin was originally isolated from human pregnancy urine by a procedure that included lectin affinity chromatography (11). This distinction is also significant because THP and uromodulin are often used synonymously in many studies (22). It is abundantly clear from the present study that uromodulin isolated from human pregnancy urine represents a discrete set of THP glycoforms that are different from those expressed in nonpregnant females and males.

The present results provide convincing evidence that both uromodulin and THP express O-glycans. This observation differs from the result of a previous study suggesting that THP or uromodulin are devoid of such sequences (23). One possible explanation for this discrepancy could be the imprecise methods employed in this previous investigation. Afonso and coworkers digested THP with pronase to generate glycopeptides that were then analyzed on gel filtration columns. It is very difficult to obtain exact structural definition using such an approach. Moreover, at the time that their study was performed, O-glycan expression was often detected by measuring the amount of GalNAc present in glycopeptide fractions. The expression of the Sd⁰ antigen on the terminal ends of N-glycans associated with both uromodulin and THP precluded this monosaccharide analysis of glycopeptides as an indicative method for detecting O-glycan expression.
The N-linked oligosaccharides associated with THP and uromodulin have been the focus of numerous structural studies employing definitive biophysical analysis (21, 24-27). There are eight potential N-linked glycosylation sites, of which seven (Asn52, Asn56, Asn208, Asn251, Asn298, Asn372, and Asn489) are occupied (27). Biantennary, triantennary and tetraantennary complex type glycans constitute most of the N-linked oligosaccharides. High mannose type glycans are attached at only one site (Asn251), where they represent 67% of the total glycans linked at this position (27). The complex type glycans are terminated with the following antennae attached to core mannosyl residues: Galβ1-4GlcNAc, NeuAcα2-3Galβ1-4GlcNAc, NeuAcα2-6Galβ1-4GlcNAc, Gal3Sβ1-4GlcNAc, and GalNAc4Sβ1-4GlcNAc (21). Polylactosamine type sequences are also attached to some of the N-glycans associated with THP (28). However, this expression is a donor specific feature not found on all samples of this glycoprotein (25).

Rigorous methods of biophysical analysis have now been used to confirm that O-glycans are also linked to THP. Both males and non-pregnant females express very simple mono- and disialylated derivatives of core 1 type sequences on THP. By contrast, the O-glycans derived from uromodulin are primarily core 2 O-linked glycans that are further branched on the β1-3 linked Gal to generate sophisticated structures expressing up to three sialyl Lewis\(^\varepsilon\) sequences (Fig. 8). Analysis of maternal THP two months postpartum indicates that the synthesis of these unusual core 2 type O-glycans is strictly pregnancy related. This specific association of the sialyl Lewis\(^\varepsilon\) sequence with uromodulin could be particularly significant, especially given the previously reported immunomodulatory activities of this glycoprotein. The sialyl Lewis\(^\varepsilon\) antigen (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc) was originally identified as a differentiation antigen associated with neutrophils and monocytes (29,30). Several groups demonstrated that oligosaccharides and
glycolipids terminated with the sialyl Lewis\(^x\) sequence serve as ligands for E-, P-, and L-selectin mediated binding (31-35). However, only E-selectin displays preferential adhesion to the sialyl Lewis\(^x\) sequence under physiological circumstances. P-selectin binding to its primary glycoprotein ligand (PSGL-1) requires the co-presentation of the sialyl Lewis\(^x\) sequence and a specific tyrosine sulfate residue on an NH\(_2\)-terminal mucin-like domain (36,37). L-selectin forms a tighter interaction with sulfated forms of the sialyl Lewis\(^x\) sequence (38). It is noteworthy that most of the E- and L-selectin ligands on the surface of murine neutrophils are carried on core 2 O-glycans, based on results obtained with core 2 GlcNAc transferase (C2GnT) knockout mice (39).

It is also significant in this context to note that neutrophils have been shown to interact with both THP and uromodulin in \textit{in vitro} assay systems. Neutrophils specifically bind to microtiter plates coated with THP (40). This interaction is calcium dependent, requires metabolically active cells, and is inhibited by soluble THP. However, this binding was also partially inhibited by the peptide sequence YRGDG, suggesting that integrin mediated adhesions could play a role in this interaction (40). Yu and coworkers reported that THP purified from normal human pregnancy urine increases phagocytosis, complement receptor expression and arachidonic acid metabolism of neutrophils (41). These investigators suggested that this glycoprotein could play a significant role in the defense mechanisms of the urinary tract.

The data presented in this paper provide a new impetus for re-examining the immunomodulatory effects of THP versus uromodulin. In particular, the current results suggest a potential structural basis for the differential activity of uromodulin and THP in the antigen induced T cell proliferation assay system reported by Muchmore and colleagues over a decade ago (12). These investigators relied upon affinity
chromatography on Concanavalin A agarose to isolate uromodulin, whereas in this study the salt
precipitation method was employed (1). This difference could potentially impact immunological assays. On
the other hand, a very substantial percentage of uromodulin molecules express biantennary and high
mannose type glycans based on the results of previous structural analyses (21). Such THP glycoforms
would be expected to bind to Concanavalin A agarose (42). Thus differences between the current and
previous preparations of uromodulin may be minimal.

The present investigation indicates that the O-glycans of THP change radically during pregnancy.
This result infers that the glycosylation of THP could be regulated by specific pregnancy related hormones.
The systemic levels of both the estrogens and progesterone are substantially increased in gravid human
females (43,44). It is significant in this context to note that human steroid hormone receptors are present in
the kidneys (45). In baboons, progesterone receptors are localized to the epithelial cells of the thick
ascending limbs of the loop of Henle and the most proximal part of the distal convoluted tubule, the
primary site of synthesis of THP in the human kidney (46). Therefore steroid hormonal stimulation
occurring during pregnancy may specifically induce the expression of specific glycosyltransferases
required to synthesize the unusual core 2 type O-glycans linked to uromodulin. Key enzymes that could be
under hormonal regulation include the fucosyltransferase(s) essential for the synthesis of the sialyl Lewis^x
sequence (47) and the C2GnT essential for converting core 1 to core 2 type O-glycans (48). Other N-
acetylgalcosaminyltransferases essential for forming multivalent sialyl Lewis^x structures may also be
hormonally regulated in the epithelial cells that synthesize THP.
In spite of extensive investigation, the precise physiological role(s) of THP and uromodulin have not yet been determined (22). However, the pregnancy specific expression of differential THP glycoforms suggests a potential functional role during this process. In a previous study, we demonstrated that gender specific changes in the glycosylation of glycodelin, a glycoprotein synthesized in both the male and female reproductive tract, led to completely different biological activities (49,50). It is our current operating hypothesis that glycosylation plays a pivotal role in tailoring the function of a subset of glycoproteins to their gender specific roles in reproduction. Structural and functional studies are underway to determine if THP/uromodulin is yet another glycoprotein that fits into this category.
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Figure legends

Figure 1. FAB mass spectra of the reductively eliminated glycans of THP/uromodulin isolated from a single male. Glycans were permethylated and subjected to Sep-Pak clean-up. (a) 35% acetonitrile fraction, (b) 50% acetonitrile fraction. Signals are assigned in Table 1. The A-type fragment ion at m/z 1070 is most likely derived from “contaminating” N-glycans (see text) which are known to be rich in the Sda sequence (21).

Figure 2. FAB mass spectra of the reductively eliminated glycans of THP/uromodulin isolated from a single female. Glycans were permethylated and subjected to Sep-Pak clean-up. (a) 35% acetonitrile fraction, (b) 50% acetonitrile fraction. The signals at m/z 873 and m/z 1234 represent the protonated form of m/z 895 and m/z 1256 respectively. The signals at m/z 897, m/z 947, m/z 1242 and m/z 1258 are contaminants.

Figure 3. FAB mass spectra of the reductively eliminated glycans of THP/uromodulin isolated from a single pregnant female. Glycans were permethylated and subjected to Sep-Pak clean-up. (a) 50% acetonitrile fraction during pregnancy, (b) 50% acetonitrile fraction 2 months post-partum. The minor
signals near m/z 2600 and 3300 in Fig 3b correspond to N-glycans as exemplified by the presence of twin peaks separated by 16 mass units.

Figure 4. FAB mass spectra of the reductively eliminated glycans of THP/uromodulin isolated from a single pregnant female following permethylation and Sep-Pak clean-up. (a) 35% acetonitrile fraction during pregnancy, (b) 35% acetonitrile fraction 2 months after pregnancy. The signals at m/z 901 and m/z 973 in (a) are contaminants.

Figure 5. FAB mass spectra of the reductively eliminated glycans of THP/uromodulin isolated from a single pregnant female before and after treatment with hydrogen fluoride. Both samples were permethylated and cleaned-up by Sep-Pak. (a) 50% acetonitrile fraction prior to treatment, (b) 50% acetonitrile fraction following treatment. The signals at m/z 901, m/z 1105, m/z 1211 and m/z 1309 are contaminants.

Figure 6. FAB mass spectra of the reductively eliminated glycans of uromodulin upon treatment of glycopeptides with Arthrobacter ureafaciens sialidase. Following the reaction the glycans were released from the glycopeptides by reductive elimination, permethylated and purified by Sep-Pak. (a) uromodulin glycans prior to treatment with sialidase (b) uromodulin glycans after treatment with sialidase. The signals at m/z 697, m/z 901, m/z 1105, m/z 1211, m/z 1309, m/z 1513, m/z 1718 and m/z 1922 are contaminants.
Figure 7. FAB mass spectra of the reductively eliminated glycans from uromodulin following treatment with periodate. After periodate treatment the glycans were reduced, permethylated and purified by Sep-Pak. (a) 35% acetonitrile fraction (b) 50% acetonitrile fraction. The majority of signals are assigned in Table 3. Signals at m/z 1552, 1757 and 1961 correspond to reduced high mannose glycans (Man_5 to Man_7 respectively) whose terminal GlcNAcitol has been cleaved between carbons 5 and 6 by the periodate treatment. The signal at m/z 1800 corresponds to residual Man_6GlcNAcGlcNAcitol.

Figure 8. Proposed structures of the unusual O-glycans present on uromodulin. Linkage analysis indicates that the branched Gal is substituted at positions 3 and 6.

Table legends

Table 1. Assignments of molecular([M+Na]^+) and A-type fragment ions detected in the FAB mass spectra of permethylated glycans derived from three male (M1-M3) and three female (F1-F3) THP samples. Major components are shown by ticks and minor components by bracketed ticks. The dashes indicate the absence of the relevant peak in the spectrum. Asterisked signals correspond to N-glycans.

Table 2. Assignments of the molecular([M+Na]^+) and A-type fragment ions detected in the FAB mass spectra of permethylated glycans derived from uromodulin. Major components are shown by ticks and
minor components by bracketed ticks. The dashes indicate the absence of the relevant peak in the spectrum. Asterisked signals correspond to N-glycans.

Table 3. Assignments of molecular ions ([M+Na]+) of reductively eliminated uromodulin O-glycans following periodate treatment, reduction and permethylation. See text for explanation of C2 and C4. Note that each of the NeuAc residues lacks carbons 8 and 9 as a result of the periodate cleavage.

Table 4. Data from linkage analysis of the partially methylated alditol acetates obtained from reductively eliminated glycans of uromodulin. The 50% acetonitrile fractions from Sep-Pak purifications of permethylated glycans were hydrolysed, reduced, acetylated and analysed by GC-MS.
| Signal (m/z) | Composition                  | M1 | M2 | M3 | F1 | F2 | F3 |
|------------|-----------------------------|----|----|----|----|----|----|
| 534        | HexHexNAc<sub>R</sub>       | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |
| 779        | HexHexNAcHexNAc<sub>R</sub> | -  | -  | -  | ✓  | ✓  | ✓  |
| 895        | NeuAcHexHexNAc<sub>R</sub>  | ✓  | ✓  | ✓  | ✓  | ✓  | -  |
| 953        | FucHexHexNAcHexNAc<sub>R</sub> | ✓  | ✓  | -  | -  | -  | -  |
| 983        | Hex<sub>3</sub>HexNAcHexNAc<sub>R</sub> | ✓  | ✓  | ✓  | -  | ✓  | ✓  |
| 1070       | NeuAcHexHexNAc<sub>2</sub>^+ | ✓  | -  | -  | ✓  | ✓  | -  |
| 1140       | NeuAcHexHexNAcHexNAc<sub>R</sub> | ✓  | ✓  | ✓  | -  | -  | -  |
| 1157       | Fuc<sub>3</sub>HexNAcHexNAc<sub>R</sub> | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |
| 1256       | NeuAc<sub>2</sub>,HexHexNAc<sub>R</sub> | ✓  | ✓  | ✓  | ✓  | -  | -  |
| 1331       | Fuc<sub>2</sub>Hex<sub>2</sub>HexNAcHexNAc<sub>R</sub> | ✓  | ✓  | ✓  | -  | -  | -  |
| 1344       | NeuAc<sub>2</sub>,HexNAcHexNAc<sub>R</sub> | ✓  | ✓  | ✓  | -  | -  | -  |
| 1484*      | Hex<sub>2</sub>HexNAc<sup>+</sup> | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |
| 1580*      | Hex<sub>2</sub>,HexNAc<sub>2</sub> | ✓  | -  | -  | ✓  | ✓  | ✓  |
| 1596*      | Hex<sub>2</sub>HexNAcHexNAc<sub>R</sub> | ✓  | ✓  | -  | ✓  | ✓  | ✓  |
| 1688*      | Hex<sub>2</sub>,HexNAc<sup>+</sup> | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |
| 1784*      | Hex<sub>2</sub>,HexNAc<sub>2</sub> | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |
| 1800*      | Hex<sub>3</sub>HexNAcHexNAc<sub>R</sub> | ✓  | ✓  | ✓  | ✓  | ✓  | ✓  |
| 1836*      | Fuc<sub>3</sub>HexNAc<sub>4</sub> | -  | -  | -  | ✓  | -  | -  |
| 1852*      | Fuc<sub>3</sub>HexNAc<sub>4</sub>,HexNAc<sub>R</sub> | -  | -  | ✓  | -  | -  | -  |
| 1988*      | Hex<sub>2</sub>,HexNAc<sub>2</sub> | ✓  | -  | -  | ✓  | ✓  | ✓  |
| 2004*      | Hex<sub>3</sub>HexNAcHexNAc<sub>R</sub> | ✓  | -  | -  | ✓  | ✓  | ✓  |
Table 2

| Signal (m/z) | Composition                          | Pregnant female 1 | Pregnant female 2 |
|-------------|--------------------------------------|-------------------|-------------------|
| 534         | HexHexNAc<sub>R</sub>                | ✓                 | ✓                 |
| 779         | HexHexNAcHexNAc<sub>R</sub>          | ✓                 | -                 |
| 895         | NeuAcHexHexNAc<sub>R</sub>           | ✓                 | ✓                 |
| 953         | FucHexHexNAcHexNAc<sub>R</sub>       | ✓                 | ✓                 |
| 983         | Hex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1140        | NeuAcHexHexNAcHexNAc<sub>R</sub>     | ✓                 | ✓                 |
| 1157        | FucHex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1256        | NeuAc<sub>R</sub>HexHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1331        | Fuc<sub>R</sub>Hex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1344        | NeuAcHex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1519        | NeuAcFucHex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1580*       | Hex<sub>R</sub>HexNAc<sub>R</sub>   | ✓                 | ✓                 |
| 1596*       | Hex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1693        | NeuAcFucHex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1784*       | Hex<sub>R</sub>HexNAc<sub>R</sub>   | ✓                 | ✓                 |
| 1800*       | Hex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1880        | NeuAc<sub>R</sub>FucHex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1938        | NeuAcFuc<sub>R</sub>Hex<sub>R</sub>HexNAc<sub>R</sub> | ✓                 | ✓                 |
| 1988*       | Hex<sub>R</sub>HexNAc<sub>R</sub>   | ✓                 | ✓                 |
| 2004*       | Hex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 2142        | NeuAcFuc<sub>R</sub>Hex<sub>R</sub>HexNAc<sub>R</sub> | ✓                 | ✓                 |
| 2504        | NeuAc<sub>R</sub>FucHex<sub>R</sub>HexNAcHexNAc<sub>R</sub> | ✓                 | ✓                 |
| 2748        | NeuAc<sub>R</sub>FucHex<sub>R</sub>HexNAc<sub>R</sub> | ✓                 | ✓                 |
| 2952        | NeuAc<sub>R</sub>FucHex<sub>R</sub>HexNAc<sub>R</sub> | ✓                 | ✓                 |
| 3126        | NeuAc<sub>R</sub>FucHex<sub>R</sub>HexNAc<sub>R</sub> | ✓                 | -                 |
| 3313        | NeuAc<sub>R</sub>FucHex<sub>R</sub>HexNAc<sub>R</sub> | ✓                 | ✓                 |
| 3487        | NeuAc<sub>R</sub>FucHex<sub>R</sub>HexNAc<sub>R</sub> | ✓                 | ✓                 |
Table 3

| Mass (m/z) | Composition                      |
|------------|----------------------------------|
| 35% acetonitrile fraction |                                |
| 562        | HexHexNAc-C2                      |
| 719        | NeuAcHex-C4                       |
| 736        | FucHexHexNAc-C2                   |
| 835        | NeuAcHexHexNAc-C2                 |
| 1009       | NeuAcFucHexHexNAc-C2              |
| 1069       | FucHex$_2$HexNAc-C4               |
| 1342       | NeuAcFucHex$_2$HexNAc-C4          |
| 50% acetonitrile fraction |                                |
| 1587       | NeuAcFucHex$_2$HexNAc$_2$-C4      |
| 1791       | NeuAcFucHex$_3$HexNAc$_2$-C4      |
| 1967       | NeuAcFuc$_2$Hex$_2$HexNAc$_2$-C4  |
| 2065       | NeuAc$_2$FucHex$_2$HexNAc$_2$-C4  |
| 2240       | NeuAc$_2$Fuc$_3$Hex$_3$HexNAc$_2$-C4 |
## Table 4

| Elution time (mins) | Characteristic fragment ions | Assignment |
|---------------------|------------------------------|------------|
| 17.38\(^a\)         | 102, 115, 118, 131, 162, 175 | Terminal fucose |
| 19.00               | 102, 118, 129, 145, 161, 162, 205 | Terminal mannose |
| 19.29\(^e\)         | 102, 118, 129, 145, 161, 162, 205 | Terminal galactose |
| 20.22               | 129, 130, 161, 190           | 2-linked mannose |
| 20.54\(^d\)         | 101, 118, 161, 234           | 3-linked galactose |
| 21.25\(^f\)         | 118, 129, 143, 185, 203      | 3,4-linked galactose |
| 21.64               | 88, 130, 246, 290            | 3-linked GalNAcitol |
| 21.92               | 129, 130, 189, 190           | 2,6-linked mannose |
| 22.10               | 118, 129, 189, 234           | 3,6-linked mannose |
| 22.29\(^c\)         | 118, 129, 189, 234           | 3,6-linked galactose |
| 23.15\(^f\)         | 117, 143, 145, 159, 203, 205 | Terminal GlcNAc |
| 23.57\(^c\)         | 88, 101, 130, 246, 318       | 3,6-linked GalNAcitol |
| 23.65\(^f\)         | 117, 143, 145, 159, 203, 205 | Terminal GalNAc |
| 24.09\(^b\)         | 117, 159, 233               | 4-linked GlcNAc |
| 24.99\(^a\)         | 117, 159, 346               | 3,4-linked GlcNAc |

\(^a\) lost after HF treatment; \(^b\) increased after HF treatment; \(^c\) decreased post-partum; \(^d\) decreased after sialidase digestion; \(^e\) increased after sialidase digestion; \(^f\) very minor component
Figure 8

= Sialic acid  ▼ = Fucose  □ = GlcNAc  ■ = GalNAc  ● = Galactose
Pregnancy associated changes in the glycosylation of Tamm-Horsfall glycoprotein. Expression of sialyl Lewisx sequences on core 2 type O-glycans derived from uromodulin

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