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# Glycosylation and over-expression of endometriosis-associated peritoneal haptoglobin

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**Peritoneal endometriotic tissues synthesize and secrete haptoglobin (pHp), which has an analogous nucleotide sequence to hepatic haptoglobin found in serum (sHp). This study performed enzymatic digestions and lectin binding assays to determine if differences in protein glycosylation exist between sHp and pHp, which may provide insight into pHp function and/or identify epitopes for development of novel methods of medical management of endometriosis. To reduce the dependence on surgical collection of peritoneal tissues from women, recombinant peritoneal Hp (rpHp) was produced and its glycosylation analyzed for future functional studies. These results showed the apparent molecular weight of pHp was 3 kDa smaller than sHp. Desialylation and complete N-deglycosylation elicited similar shifts in sHp and pHp electrophoretic migration, suggesting similar sialic acid content and indicating the 3 kDa variance was due to carbohydrate content, not protein degradation, respectively. Sequential deglycosylation of the four sHp N-glycan chains caused a 3 kDa shift per N-glycan removed suggesting the 3 kDa difference between sHp and pHp may be one N-glycan chain. Lectin ELISA and lectin-blotting analyses demonstrated increased pHp and rpHp interactions with MAL and LTL but no difference in binding to SNL compared to sHp from healthy individuals, identifying variations in the ratios of $\alpha$(2-3) to $\alpha$(2-6) sialic acid and fucose residues. Recombinant pHp was 100-fold over-expressed with a similar glycosylation pattern to pHp, albeit in an unprocessed $\alpha$-$\beta$ Hp polypeptide form. These results are the first to identify differences between pHp and sHp glycosylation and lay groundwork further studies to characterize anomalies in glycan composition and structure, which likely impart pHp with known immunomodulatory functions and may be used as epitopes for development of immune based therapeutics for novel, non-surgical management of endometriosis.**

***Keywords:*** **peritoneal endometriosis, peritoneum, haptoglobin, recombinant haptoglobin, glycosylation**

## Introduction

### Endometriosis

Endometriosis is a common gynecological disorder defined by the presence of lesions composed of endometrial glands and stroma located outside the uterine cavity [1–4]. It causes chronic, intractable pelvic pain and infertility in women of reproductive age. Diagnosis of endometriosis requires costly, invasive surgery to identify the endometriotic lesions; subsequent treatment may be surgical or medical therapy, both aimed at elimination of the lesions [4]. Endometriosis cannot, however, be cured by any known treatment.

Sampson's theory of retrograde menstruation provides a plausible explanation for the presence of endometrial tissue in the peritoneal cavity [1] and characteristics of established endometriotic lesions have been described [1–4]. To date, however, limited progress has been made toward understanding the pathogenesis of endometriosis. Defining the cellular and molecular pathogenic mechanisms of endometriosis may hold the key to developing new non-surgical methods for diagnosis and treatment of this disorder.

Intriguingly, our research has shown that established peritoneal endometriotic lesions synthesize and secrete the acute phase response protein haptoglobin (Hp), both *in vivo* and *in vitro* [5–9]. Further characterization of peritoneal endometriotic Hp (pHp) may provide insight into mechanisms which support known pHp immunomodulatory functions involved in the pathogenesis of endometriosis [10] and, by comparing it to hepatic Hp found in the serum Hp (sHp), will help determine the utility of pHp as a novel alternative, non-invasive method for medical management of endometriosis.

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### Hepatic haptoglobin

The liver is the most well-known and main site of hepatic Hp production. Hepatic Hp is normally found in the serum (sHp) at concentrations between 1 to 3 mg/ml. Hepatic Hp is a hetero-tetramer consisting of two $\alpha$-subunits and two-glycosylated $\beta$-subunits joined by inter-chain disulfide bonds [11]. The carbohydrate content of hepatic Hp is $\sim$20% and is found external to the protein, exclusively as N-linked, complex oligosaccharide units called glycans. These occur at four distinct asparagine residues (ASN 23, 46, 50, 80) of each of the $\beta$-chains. They may include fucose, galactose, mannose, N-acetyl-glucosamine and sialic acid [11–13]. Hepatic Hp displays heterogeneity in glycosylation. The ratio of $\alpha$(2-6) to $\alpha$(2-3) linked sialic acid is about 4:1, the latter are found on the triantennary chains [12]. Approximately 17% of the glycans are monosialylated, biantennary glycans. Interestingly, unique and characteristic changes in fucose content, sialic acid content and linkage from $\alpha$(2-6) to $\alpha$(2-3) and glycan branching define several diseases, as well as novel modifications in Hp function [10,14–18].

Inflammatory cytokines such as interleukin-6 (IL-6) significantly alter glycosylation of acute phase response proteins synthesized by hepatocytes and human hepatoma cell lines, including Hp [19]. These studies have shown that IL-6 modifies glycan branching, but less is known about its effects on terminal carbohydrates [20]. Evidence suggests that inflammation- or disease-induced alterations in terminal fucosylation and sialylation are associated with pathophysiological variations in glycoforms of acute phase response proteins [19]. Moreover, the degree of glycan branching and the type of carbohydrates present on acute phase response proteins depend on the cell type in which they are produced, and profoundly affect the functional properties of the protein [19].

### Endometriosis-associated haptoglobin

The nucleotide sequences of sHp and pHp are analogous [7], both are composed of $\alpha$- and $\beta$-subunits, and the total concentration of Hp in the serum does not vary between women with and without endometriosis [21], eliminating their potential for diagnostic distinction. Importantly, identification of unique pHp glycan moieties may provide the required specificity and sensitivity to differentiate pHp from sHp for utility as a clinical marker for endometriosis. To our knowledge, pHp glycans have not yet been investigated.

*In vitro* synthesis and secretion of pHp by endometriotic lesion cells and by peritoneal cells is dramatically up-regulated by treatment with IL-6, a cytokine associated with the pathogenesis of endometriosis [8]. Ectopic endometriotic cells and eutopic endometrial cells from women with endometriosis actually produce significant amounts of IL-6 *in vitro* as compared to endometrial cells from healthy individuals [8,22]. It is possible that, in women with endometriosis, IL-6 production by endometrial and endometriotic cells stimulates expression and alters glycosylation of Hp produced by endometriotic lesions and peritoneal cells.

The most studied biological function of hepatic Hp is the recapture of hemoglobin; yet, Hp has other biological activities distinct from this role [23,24]. We have previously reported that Hp from endometriotic lesions binds to immune cells and alters their function by decreasing their adherence to foreign substrates and increasing IL-6 secretion [10]. The mechanism of pHp-macrophage interaction has not been defined, but could involve pHp glycosylation and specific integrin receptors present on some immune cells [18,25]. As anomalies of immune cell function are believed to be central to the pathogenesis of endometriosis [26], further characterization of the glycosylation of endometriosis-associated Hp is warranted.

Therefore, to determine if glycosylation differences existed between pHp and sHp, this study performed specific glycosylation analyses by enzymatic digestion and lectin binding assays. Also due to limitations associated with surgical collection of peritoneal tissues in women combined with the need for substantial amounts of correctly glycosylated pHp for future functional studies, a strategy was developed for over-expression of recombinant peritoneal Hp (rpHp) in homologous peritoneal cells transfected with Hp cDNA and the glycosylation of the rpHp product evaluated.

## Materials and methods

Informed consent was obtained from all human subjects as approved by the University of Missouri Institutional Review Board—Health Sciences Section.

### Serum haptoglobin (sHp)

Blood samples, collected as a source of normal sHp, were obtained by venipuncture from healthy female volunteers ($n = 5$) without pelvic disease as assessed during laparoscopy. These women were non-smokers, non-drinkers and were not taking any hormone-modulating medication. The sera were separated by low speed centrifugation (600$\times$ g) and stored at $-80^{\circ}$C. Serum Hp form normal individuals was used as the standard to compare pHp glycosylation.

### Endometriosis-associated peritoneal haptoglobin (pHp)

Endometriotic lesions ($n = 5$) and non-affected serosal peritoneal tissues ($n = 2$) were obtained from subjects with a clinical and histopathological diagnosis of endometriosis as previously described [5]. Cells were obtained from tissues by enzymatic digestion and the adherent cells cultured as previously reported [8]. Pre-confluent cells were cultured at 37$^{\circ}$C with 5% $CO_2$ in phenol red-free DME/Ham's F12 medium (DME/F12; Sigma) containing 10% heat-inactivated fetal bovine serum (FSB, Life Technologies, Grand Island, NY), 100 IU/ml of penicillin and 100 mg/ml of streptomycin (Sigma). As previously described and validated [8], replicate cell cultures were evaluated by cytokeratin (epithelial cell intermediate filament marker, Biodesign, Kennebunk, ME) and

vimentin (stromal cell intermediate filament marker, Roche, Indianapolis, IN) immunohistochemical staining.

To enhance Hp expression, near confluent peritoneal cell cultures were cultured with serum-free medium for 48 hr and then exposed to 1 $\mu$M DEX (Sigma, St. Louis, MO) for 14 hr. Fresh serum-free medium containing 1 $\mu$M DEX and 25 ng/ml human recombinant IL-6 (DEX/IL-6) was added every five days for up to one month. As peritoneal endometriotic lesion cells produce high amounts of endogenous IL-6 [8] neither DEX nor endogenous IL-6 was added to these cultures.

### Cloning of endometriotic lesion Hp cDNA and over-expression of recombinant Hp in primary peritoneal cell cultures

Human hepatic Hp is composed of $\alpha$- and $\beta$-subunits translated from the same mRNA, with three different $\alpha$-chain phenotypes 1-1, 2-1 and 2-2 [27]. All three phenotypes are expressed by human peritoneal endometriotic lesions [28]. Full-length endometriotic lesion Hp cDNA was cloned using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). Human peritoneal endometriotic lesion mRNA was reverse-transcribed using the synthesis primer [5′-TTCTAGAATTCAGCGGCCGC$(T)_{30}N_{-1}$N-3′]. Full-length Hp $\alpha_2$-$\beta$ and $\alpha_1$-$\beta$ cDNA were amplified with 0.05 U/$\mu$l of *Pfu* DNA polymerase (Stratagene, La Jolla, CA).

The forward primer was:

5′GGGAATTCAGAGGCAAGACCAACCAAGATGAGTG-3′ (*Eco*R I site).

The reverse primer was:

5′-GTCTAGACTTATCGTCATCGTCGTTCTCAGCTATGGTCTTCTGAACCC-3′ (*Xba* I site).

Hp $\alpha_2$-$\beta$ and $\alpha_1$-$\beta$ full-lengthcDNAs were cloned into the mammalian expression vector pcDNA6/V5-HisA (Invitrogen, Carlsbad, CA) in the corresponding *Eco*RI/*Xba*I sites and sequenced. The final expression construct included a C-terminal tag that encoded for the enterokinase cleavage site DDDK, the V5 epitope GKPIPNPLLGLDST to monitor expression and a $(His)_6$ metal-binding peptide for affinity-chromatography purification.

Primary cultures of human peritoneal cells were transfected with 10 $\mu$g of the recombinant Hp $\alpha_2$-$\beta$-pcDNA6/V5-His by calcium phosphate methodology following manufacturer's instructions (Invitrogen). Transfected cells were cultured in DME/F12 medium containing FBS, penicillin and streptomycin as described above.

### Purification and quantification of Hp

All culture media were collected every five days and stored separately at $-80^{\circ}$C prior to chromatographic Hp purification. All forms of Hp were chromatographically purified from sera and all cell culture media as previously specified [29]. The purity of all Hp preparations was routinely checked by 10% SDS-PAGE followed by silver staining. A specific Hp ELISA was used to measure Hp concentrations in cell culture media [8].

### Analysis of Hp sialic acid and N-glycan content

Sialic acid content of pHp, rpHp and sHp were evaluated by incubating 20 ng of Hp at 37°C with 10 mIU of *Arthrobacter urefaciens* sialidase (Glyko, Novato, CA) in 50 mM sodium phosphate, pH 6.0. This glycosidase releases $\alpha$(2-3), $\alpha$(2-6), $\alpha$(2-8), $\alpha$(2-9) linked sialic acid from complex carbohydrates. Desialylation efficiency was monitored by lectin blotting analysis with biotinylated *Sambucus nigra* lectin (SNL, Vector Laboratories, Burlingame, CA), which binds to sialic acid, terminally linked $\alpha$(2-6) to galactose or N-acetyl galactosamine [30].

Total N-glycan content of pHp, rpHp and sHp were evaluated by incubating 80 ng of Hp with 2 mIU of recombinant *Flavobacterium meningosepticum* N-glycanase (Glyko), which cleaves intact N-linked glycans from glycoproteins. Additionally, sequential N-glycosylation analyses of sHp were performed by treatment with N-glycanase for 2, 15, 30, 60 min and 18 hr. The sequential N-glycosylation analyses of native pHp were not performed, resorting to selective analyses of key glycans, due to limitations of enrolling large numbers of women to undergo invasive surgery as endometriotic tissue donors and the fact that purification of pico- or nanogram quantities of pHp would require peritoneal tissue biopsies from numerous women.

The resultant products of the sHp, pHp and rHp enzymatic reactions were resolved by 10% SDS-PAGE (pHp and sHp) or 8% SDS-PAGE (rpHp), transferred to nitrocellulose membranes (Micro Separations Inc., Westborough, MA) and Western blot analyses was performed with rabbit anti-human Hp (Dako, Carpentaria, CA) as previously described [6]. Apparent molecular weights were calculated by their relative mobility with respect to protein standards.

Lectin ELISA was performed as described by Goodarzi and Turner [31]. *Maackia amurensis* lectin (MAL, Vector Laboratories, recognizes sialic acid terminally linked $\alpha$(2-3) to galactose or N-acetylgalactosamine [31]), Lotus tetragonolobus lectin (LTL, Sigma, binds fucose linked $\alpha$(1-2) to galactose, $\alpha$(1-3) to peripheral N-acetylglucosamine [32,33] and $\alpha$(1-6) to the N,N′-diacetylchitobiose core [33]), and SNL were used. MAL, LTL, and SNL were used at concentrations of 10 $\mu$g/ml, 2g$\mu$g/ml, and 1 $\mu$g/ml, respectively. The background absorbance (i.e. without protein) was subtracted from all data. Absorbance was read at 405 nm and the reference wavelength was 630 nm.

Absorbance values at 405 nm (A) MAL, LTL and SNL were plotted against the amount of pHp, rpHp or sHp in ng/100 $\mu$l (c) for each independent experiment. The slope (m) for each equation $A = mc$ was calculated using the Excel program (Microsoft Corporation, Redmond, WA). These slope values, which represent the amount of lectin bound by one ng of Hp, were normalized by square root transform when necessary (LTL ELISA) and compared by Student's $T$-test.

Lectin binding was also assessed using the DIG Glycan Differentiation kit from Roche (Indianapolis, IN) following manufacturer's instructions. Interactions of Hp and MAL, LTL and SNL were evaluated.

## Results

### Cell cultures

Greater than 95% of the peritoneal and endometriotic lesion cells used to produce pHp strongly reacted with the vimentin antibody but not the cytokeratin antibody as previously reported [8]. Adherent endometrial and peritoneal cell preparations obtained from similar enzymatic digestions and cultured for more than 6 days have been previously characterized to be free of endothelial cells (anti-Factor VIII immuno-negative) and leukocytes (anti-CD 45 immuno-negative) [5,34]. Morphologically, the cells were spindle-shaped and elongated and appeared as a single cell monolayer. A few clusters of polygonal-shaped cells stained for cytokeratin intermediate filaments and were, thus, of epithelial/mesothelial cell origin. These results indicate that the vimentin positive cells used in these experiments consisted basically of cells from mesenchymal origin.

### Production of pHp and cloning and expression of rpHp

Production of Hp by non-stimulated, non-transfected peritoneal cells ($n = 2$) was below the ELISA detection limit (0.25 ng/ml) [8]. Endometriotic lesion cells or IL-6 stimulated peritoneal cells ($n = 7$) produced $0.014 \pm 0.002$ $\mu$g of pHp $\times$ $(10^6$ cells$)^{-1} \times (48$ h$)^{-1}$.

Hp $\alpha_2$-$\beta$ and $\alpha_1$-$\beta$ cDNAs isolated from human endometriotic lesions were identical to hepatic Hp [35] and contained the signal peptide for secretion and a non-canonical Kozak sequence (AAGATGAG). Transfected peritoneal cells ($n = 2$) secreted $1.4 \pm 0.1$ $\mu$g of rpHp $\times$ $(10^6$ cells$)^{-1} \times (48$ h$)^{-1}$.

### Effects of desialylation and N-deglycosylation

Three independent experiments performed with pHp from either endometriotic lesion cells or DEX/IL-6 stimulated peritoneal cells showed identical results. Therefore, they will be collectively referred to as pHp throughout the Results Section.

Figure 1 represents the effects of desialylation and N-deglycosylation on the electrophoretic mobility of sHp and pHp as detected by Hp immunoblot analysis. The intact sHp $\beta$-subunit appeared as immunoreactive band at 41 kDa whereas the intact pHp $\beta$-chain was 3 kDa smaller, with an apparent molecular weight of 38 kDa. Apparent molecular weights of the completely desialylated proteins were calculated as 38 and 35 kDa for sHp and pHp, respectively. Desialylation was complete as treatment of Hp with neuraminidase yielded a product unable to react with SNL as visualized by lectin-blotting analysis (data not shown).

Complete N-deglycosylation of sHp and pHp with N-glycanase yielded products of equal size (Figure 1), whose

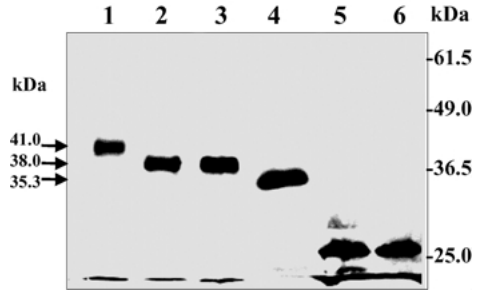


**Figure 1.** Effects of desialylation and N-deglycosylation on electrophoretic mobility of normal sHp and pHp produced under inflammatory conditions. Odd-numbered lanes correspond to sHp and even numbered lanes to pHp. Proteins were incubated without enzymes (lanes 1 and 2), with sialidase (lanes 3 and 4), or with N-glycanase (lanes 5 and 6). The proteins were resolved by SDS-PAGE, transferred to a membrane and evaluated by Western blot analyses with an antibody to human Hp. At the left of the figure, arrows indicate the apparent molecular weight of non-treated sHp $\beta$-subunit (41 kDa), non-treated pHp $\beta$-subunit (38 kDa), desialylated sHp (38 kDa) and desialylated pHp (35.3 kDa). Protein standards are indicated by their apparent molecular weight at the right of the figure.

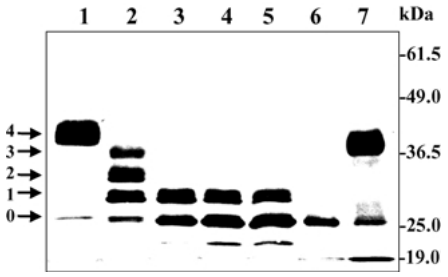


**Figure 2.** Effects of sequential N-deglycosylation and desialylation on electrophoretic mobility of sHp. sHp was incubated without enzymes for 18 hr (lane 1) or with N-glycanase for 2 min (lane 2), 15 min (lane 3), 30 min (lane 4), 60 min (lane 5) and 18 hr (lanes 6) and sialidase for 18 hr (lane 7). The proteins were resolved by SDS-PAGE, transferred to a membrane and evaluated by Western blot analyses with an antibody to human sHp. At the left of the figure, arrows indicate the number of N-glycan groups per Hp $\beta$-chain. Protein standards are indicated by their apparent molecular weight at the right of the figure.

apparent molecular weights (27 kDa) match that of the published amino acid sequence of the deglycosylated sHp $\beta$-subunit [27]. No immunoreactivity was detected when an equivalent amount of normal rabbit serum or an equivalent aliquot of an experimental blank were substituted for the Hp antibody and evaluated by anti-Hp Western blot analysis (data not shown).

Sequential N-deglycosylation of sHp was performed to evaluate shifts in apparent molecular weight that occurred as N-glycans were individually removed from sHp, eventually yielding a completely deglycosylated sHp product (Figure 2). The untreated sHp $\beta$-subunit with all four N-glycans migrated to the anticipated weight at 41 kDa. Partial digestion with N-glycanase

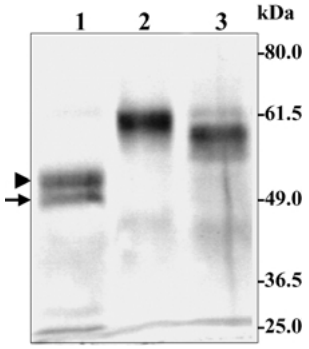


**Figure 3.** Effects of desialylation and N-deglycosylation on electrophoretic mobility of rpHp. After incubating the protein with N-glycanase (lane 1), without enzymes (lane 2) and with sialidase (lane 3), protein mixtures were resolved by SDS-PAGE, transferred to a membrane and evaluated by Western blot analyses with an antibody to human Hp. The arrow and arrowhead indicate a completely and a partially deglycosylated forms of the recombinant protein, respectively. Protein standards are indicated by their apparent molecular weight at the right of the figure.

over time showed four distinct glycoforms, each approximately 3 kDa smaller than the previous one. Complete N-glycanase digestion resulted in a single form of 27 kDa. These results confirm the work of others showing sHp has four N-linked oligosaccharide units of the complex type [11–13,23,24], each 3 kDa in size. Again, due to limitations in quantities of native pHp available for sequential N-deglycosylation studies, complete structural identification of the native pHp glycans was not performed.

The effects of desialylation and N-deglycosylation on the electrophoretic mobility of rpHp are shown in Figure 3. The rpHp was secreted into culture medium as a 61-kDa single polypeptide, indicating that the transfected peritoneal cells were unable to cleave the Hp $\alpha_2$-$\beta$ chain into the two separate subunits. No signal was detected at 38 kDa, as endogenous Hp production by these cells was at least 100 fold lower. Desialylation decreased the apparent molecular weight by about 3 kDa as compared to the non-treated protein. N-glycanase treatment yielded a protein of the expected size at 49 kDa, which is comprised of the 45-kDa Hp $\alpha_2$-$\beta$ polypeptide and a 4-kDa peptide encoded by the C-terminal tag.

### Lectin analyses

As assessed by lectin ELISA, pHp bound significantly more MAL and LTL compared to sHp from healthy individuals (Figure 4). Binding to SNL was, however, similar for pHp and sHp (Figure 4).

Lectin-blotting analysis comparing binding of rpHp and sHp showed that, like pHp, only rpHp reacted with MAL and LTL, while both sHp and rpHp reacted with SNL (Figure 5). This result was confirmed by lectin ELISA, where rpHp bound significantly more MAL and LTL than sHp (Figure 6).

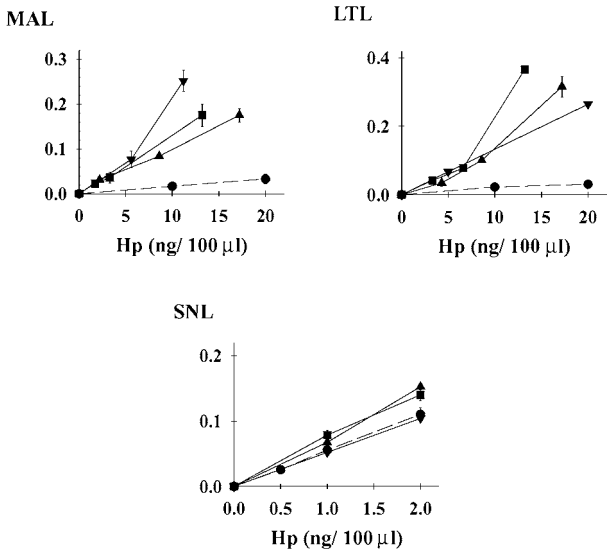


**Figure 4.** Lectin ELISA of pHp produced under the influence of dexamethasone and interleukin-6 (DEX/IL-6; solid lines, three independent experiments represented as ▼, ▲, and ■) and sHp from healthy individuals (average of five independent experiments, dashed line with ●). Hp in each sample was affinity-purified to homogeneity. All values are the means ± SEM of duplicate measurements. Significantly more pHp produced under the influence of DEX/IL-6 bound to MAL and LTL in comparison with normal sHp. Binding of pHp to MAL and LTL was 10- and 11-times greater, respectively, than sHp ($P < 0.001$). No differences were observed between pHp and sHp binding to SNL, suggesting no differences in content of sialic acid terminally linked $\alpha$2-6 to galactose ($P > 0.05$).

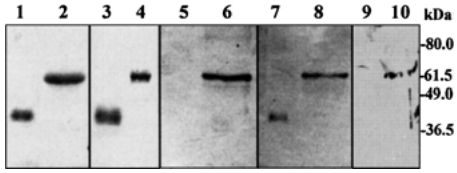


**Figure 5.** Lectin-blotting analysis of sHp and rpHp 2-2 expressed in peritoneal cells. sHp is shown in odd-numbered lanes and rpHp is shown in even-numbered lanes. rpHp was secreted as a single $\alpha_2$-$\beta$ polypeptide and affinity-purified to homogeneity as visualized by silver stained SDS-PAGE (lanes 1 and 2) and Western blot analyses (lanes 3 and 4). Lectin-blotting analysis shows that only rpHp stained with MAL (lanes 5 and 6) and LTL (lanes 9 and 10) while both rpHp and sHp reacted with SNL (lanes 7 and 8). Protein standards are indicated by their apparent molecular weight at the right of the figure.

## Discussion

These results report the discovery and initial characterization of differences in endometriosis-associated Hp protein glycosylation versus hepatic Hp glycosylation. This information provides insight into pHp glycosylation which may be involved in the mechanisms of pHp immunomodulation in the pathogenesis of endometriosis and identifies the presence of pHp glycosylation

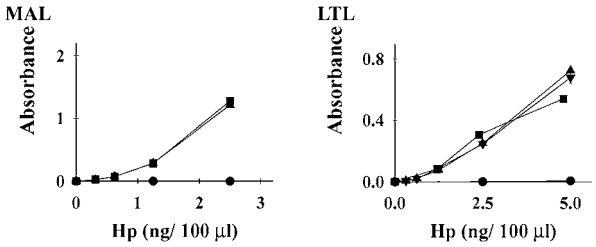


**Figure 6.** Lectin ELISA of rpHp (solid lines, two and three independent experiments for MAL and LTL, respectively, represented by ▼, ▲, and ■) and sHp from healthy individuals (average of three independent experiments represented by ●). Each sample was affinity-purified to homogeneity. All values are the means ± SEM of duplicate measurements. rpHp bound significantly more MAL and LTL than sHp ($P < 0.001$).

epitopes that may be used in development of novel, non-invasive immuno-diagnostics or therapeutics for endometriosis.

Under these experimental conditions designed to mimic an inflammatory response [20], the pHp $\beta$-chain was 3 kDa smaller than the sHp $\beta$-chain. Several explanations may account for the observed 3 kDa difference between pHp and sHp. Equivalent changes in SDS-PAGE electrophoretic migration of pHp and sHp after desialylation indirectly suggest the sialic acid content of these two glycoproteins is similar. Complete N-deglycosylation proves that the 3kDa difference between pHp and sHp was in carbohydrate content and not due to protein degradation. The sequential deglycosylation of sHp implies that pHp may lack a complete 3 kDa N-glycan unit an alteration reported in sHp isolated from patients with carbohydrate-deficient glycoprotein syndrome type I [36].

The lectin binding studies provide further insight into the glycan profiles of pHp and rpHp. The increased interactions of pHp and rpHp with MAL provide evidence of an increase in $\alpha$(2-3) linked sialic acid residues in these two glycoproteins as compared to the normal 4:1 ratio of $\alpha$(2-6) to $\alpha$(2-3) linked sialic acid of sHp [37,38]. These changes are likely to occur on triantennary chains because $\alpha$(2-3)-sialyltransferase shows a much higher affinity than $\alpha$(2-6)-sialyltransferase for oligosaccharides that are part of branched lactosaminoglycan extensions [39], suggesting pHp and rpHp may have more triantennary glycan chains that sHp. Interestingly, the glycan structure of pHp resembles that of sHp isolated from ovarian cancer patients in which biantennary chains ending in $\alpha$(2-6) sialic acid are replaced by triantennary chains with terminal $\alpha$(2-3) sialic acid residues [40].

The increased binding of pHp and rpHp to LTL suggests differential fucose content or linkages in pHp and rpHp compare to sHp. sHp isolated from breast and ovarian cancer patients also shows a dramatic increase in LTL reactivity without any change in SNL binding or sialic acid content [31,40,41].

*In vivo*, several factors affect the glycosylation of serum acute phase response proteins including the particular physiological state of the individual (acute inflammation, pregnancy) and the type of disease (cancer, alcoholic liver cirrhosis) [19,29,40–43]. The glycosylation pattern also depends on the acute phase response protein itself. For example, in women with ovarian cancer, serum $\alpha_1$-protease inhibitor exhibits decreased branching and more glycan chains ending in $\alpha$(2-6) sialic acid while sHp displays increased branching and more glycan chains ending in $\alpha$(2-3) sialic acid [40].

Serum Hp from women with endometriosis was not evaluated in these experiments. It is known that serum concentrations of sHp do not differ between women with and without endometriosis [21], but the glycosylation pattern of sHp from women with endometriosis requires further characterization. Production of pHp by either peritoneal or endometriotic cells is a local effect in the peritoneal cavity, triggered by IL-6 secreted by endometrial cells and/or macrophages. We do not anticipate that glycosylation of sHp from the liver would be altered in these women, as it is in association with some other systemic disorders, but further investigation is required.

Others and we have found no difference in the concentrations of several of the acute phase response proteins, including Hp, in peritoneal fluid of women with endometriosis [10,44], however, the glycosylation of these proteins has not been defined in endometriosis. Peritoneal fluid from women with endometriosis contains numerous inflammatory cytokines, some of which are produced by the endometriotic lesions including IL-6 [22] that could alter the glycosylation of pHp. Further, endometriotic lesions are composed of endometrial and peritoneal cells plus resident immune cells and endothelial cells. Possible interactions with the cells or their products the intact endometriotic lesion may also alter the glycosylation of endometriotic Hp.

*In vitro*, the cell type (i.e. hepatoma cell line versus hepatocytes) and the cytokine that induces the acute phase response protein are major contributory factors to glycan structure [19,20]. Studies of primary cultures of hepatocytes have clearly shown that these cells produce all the Hp glycosylation variants present in normal and patient sera. Yet in endometriosis, peritoneal cells only produce pHp when exposed DEX/IL-6 and this pHp is differentially glycosylated as compared to sHp produced by liver cells. As previously mentioned, the fact that IL-6 is known to modify glycan branching [20] supports our evidence that IL-6 stimulated pHp may have more triantennary N-glycan chains than sHp.

Our over-expression strategy using transfected primary cultures of peritoneal cells was successful in producing 100 fold more rpHp than production of pHp by DEX/IL-6 treated peritoneal cells. In support of this model, the host peritoneal cells had a homologous genetic background with the peritoneal component of endometriotic lesion cells. They produced the atypical glycosylation pattern of pHp, deemed to have alterations in the N-glycan chains with more terminal $\alpha$(2,3)-linked sialic acid and core fucose as compared to sHp, and both displayed increased binding to MAL and LTL.

This approach should, however, be used with caution. The fact that the single $\alpha$-$\beta$ polypeptide rpHp is not processed into

the separate $\alpha$- and $\beta$-subunits, possibly due to saturation of specific intracellular protease(s) involved in the Hp maturation process [45], raises the concern that the glycosylation mechanisms may also be affected in this system. Others have shown that the transfection process [46] or mutations [47] can activate latent glycosyltransferases. Curiously, the glycosylation profiles of pHp produced in DEX/IL-6 treated peritoneal cells and of rpHp produced in peritoneal cells transfected with the Hp gene but without DEX/IL-6 treatment were similar. Further experiments are needed to evaluate the relationship of DEX/IL-6 treatment and glycosyltransferase production associated with transfection and their effects on the overall glycosylation profile of pHp and rpHp. Additionally, it must be determined if over-expressed rpHp from peritoneal cells reflects the same pathophysiological mechanisms and functional status of pHp produced by endometriosis-associated mesenchymal cells.

Collectively, our prior research [5–10,21,28] and the present study indicate that like sHp, the pHp glycosylation profile includes biantennary and triantennary chains containing fucose, galactose, mannose, N-acetylglucosamine and sialic acid; however, modifications in the sialic acid linkages and glycan branching and fucose residues exist. As others have shown that these glycans can serve as ligands for immune cell $\alpha M\beta 2$ integrin receptors (also called CD11b/CD18 and MAC-1) [18,25], we propose that the pHp glycosylation modifications are involved in the mechanism whereby endometriotic Hp binds to macrophages. The binding of endometriotic Hp to macrophages alters macrophage function by decreasing their adherence to foreign substrates and increasing IL-6 and TNF-$\alpha$ (Sharpe-Timms, unpublished observation) secretion and in turn increases endometriotic tissue endometriotic Hp production [10]. Activation of the $\alpha M\beta 2$ integrin receptor by ligands other than Hp both markedly alters macrophage phagocytic function and enhances production IL-1, IL-6 and TNF-$\alpha$ [48–52]. Further studies are currently underway to evaluate a potential endometriotic Hp glycan-macrophage-integrin mechanism in relation to altered macrophage function in endometriosis.

In conclusion, the discovery of differential glycosylation pHp, whose production by cells from the peritoneal cavity is up-regulated by IL-6, meets the goals of this study and provides the groundwork for further studies of this extrahepatic Hp in the pathogenesis of endometriosis. Further, recognition of differential glycosylation of pHp compared to sHp suggests novel epitopes exist that could be used to raise specific monoclonal antibodies, essential tools to detect different glycoforms and eventually develop non-invasive diagnostics and pursue immunotherapeutic approaches for endometriosis. And while further studies to provide a complete analysis of pHp sugar composition are warranted, efforts to purify enough pHp to perform these studies must address the ethical concerns of performing significant numbers of invasive surgeries in women to collect endometriotic tissue. At present, while providing a convenient source of abundant protein possessing the same glycosylation profile of pHp, the utility of rpHp for functional studies in the pathogenesis of endometriosis remains to be defined.

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