Glycosylation & Disease, 1994, **1**, 37–43

Changes in the fucose content of haptoglobin in breast and ovarian cancer: association with disease progression

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There is increasing evidence for changes in fucosylation in cancer. Previously, we showed that the fucose-specific lectin, *Lotus tetragonolobus*, extracts an abnormal form of haptoglobin (Hp) from cancer sera. This study investigates the monosaccharide content of Hp obtained from women with ovarian and breast cancer at different stages of their disease. In both cancers, Hp fucose was low when the disease was benign or in remission and much higher when the disease was progressive. This occurred whether the data was expressed per mole of protein or per three mannose residues. Changes in other monosaccharides were minor compared with fucose. There were small increases in the *N*-acetylglucosamine and galactose content (per three mannoses) in ovarian cancer, suggesting that some glycan chains have increased branching. The latter was independent of disease activity which may be due to some indirect cause such as cytotoxic therapy or an inflammatory response. When ovarian cancer patients were in remission, the number of glycosylation sites on Hp was reduced. Hp isolated from patients with early, but not advanced breast cancer also appeared to have increased glycan branching. The increased fucosylated Hp may interfere with fucose-mediated adhesion reactions of cancer cells.

Keywords: breast cancer, fucose, haptoglobin, ovarian cancer

Introduction

Haptoglobin (Hp) [1] is a serum glycoprotein that binds avidly to haemoglobin (Hb) which is released by intravascular red cell destruction. The Hp/Hb complex is then rapidly broken down by the lymphoreticular system. This process serves to conserve iron stores and also protects the kidney from damage by the Hb. The normal concentration of Hp in the blood is 1 to 2 g/l, but in infection, inflammation and malignancy it can increase many fold.

In a previous study we showed that an abnormal form of Hp can be extracted from cancer sera by the fucose-specific lectin, *Lotus tetragonolobus* [2]. Later studies showed that this substance was a cancer marker of tumour burden but not gross liver metastasis [3], and that the concentration of this substance in the blood was correlated with the activity of α1,3 fucosyl transferase [4]. *Lotus*-extractable Hp has also been found in high amounts in active rheumatoid arthritis [5] and alcohol liver disease [6], but in some other diseases it seems to be absent [2], low [6] or present in amounts not related to disease activity [7].

Hp is composed of two α and two β subunits [1]. The carbohydrate on Hp is found only on the β subunits and consists of four *N*-linked complex chains per subunit, with equal amounts of bi- and triantennary chains [8]. The fucose on these units is reportedly present both in the core-α (1-6) position and in a α(1-3) position on an external

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Part of this work was published in abstract form, *Glycoconjugate J* 1993; **10**; 318.

N-acetylglucosamine (GlcNAc) residue [8]. More precise details of the carbohydrate structure are unknown.

There is increasing evidence for a general change in the fucosylation of molecules in cancer. These changes have been detected in cultured neoplastic cells [9], human tumours [10] and in secreted host glycoproteins [11]. Of particular relevance to invasion and metastasis are the reported increases in the expression of fucose-containing antigens (so called Lewis antigens) (Le) on cancer cells, groupings which have been shown to interact with a new class of adhesion molecules (Selectins) on vascular endothelium [12].

In a preliminary study of purified Hp obtained from ovarian cancer patients, we have confirmed that this molecule contains increased fucosylation [13]. The aim of this study was to extend this observation by measuring the monosaccharide content of Hp isolated from women with ovarian or breast cancer at different stages in their disease.

Materials and methods

Blood specimens were obtained by venepuncture from seven healthy women, 14 women with ovarian cancer, 14 women with breast cancer and six women with benign breast disease. Sera were separated by low speed centrifugation (600 *g*) for 10 min and stored at −20°C until required for analysis. All the healthy women (median age 53 years, range 31–64) were attending a blood donor session. They were non-smokers, alcohol intake was very low and none of the group were taking oral contraceptives or any other form of medication.

Ovarian cancers were diagnosed by laporotomy (stages III/IV FIGO clarification) and confirmed by histology (serous or mucinous adenocarcinoma). All had malignant extension to the small bowel or omentum and some had distant metastases in the liver. At laparotomy, different amounts of tumour were removed; in all cases, tumour remained in the abdomen, despite attempts at total surgical debulking. When the blood specimen was obtained, 10 women were receiving Carboplatin chemotherapy, one woman was receiving Chlorambucil, two women were receiving Mitoxathrone and a final women was receiving Treosulfan.

The women with breast cancer were classified into two groups—advanced and early. The advanced group consisted of seven women who had undergone either a lumpectomy or a mastectomy 2–7 years previously. All the women had clinical signs of cancer when the specimen was collected and all had detectable metastases in multiple sites. When the specimen was obtained, six of seven women were receiving Mitoxanthrone and one woman was receiving no treatment. Six of the women were previously treated with other therapies which included Aminoglutethimide, radiotherapy and Tamoxifen. The 'early' group consisted of seven women who underwent a lumpectomy or mastectomy after the specimen was collected. All the women were found to have an infiltrative ductal adenocarcinoma, which was 14–35 mm in size (four grade II and three grade III, Bloom and Richardson classification). Tumour was found in the axillary lymph nodes in three of seven of the women. Only one of seven of women was receiving treatment, which was Tamoxifen. The benign breast group consisted of seven women with either cystic disease or fibroadenoma of the breast.

Ovarian and breast cancer patients were assessed by clinical examination and appropriate radiological and ultrasound scanning techniques. In ovarian cancer, remission was defined as the disappearance of all demonstrable disease for at least 4 weeks; progressive disease was defined as more than 30% increase in the size of any measureable lesion. Details of the different disease groups studied are summarized in Table 1. None of the patients in the disease groups were taking oral contraceptives.

Hp was extracted from serum as follows. Sheep anti-human Hp antibody (Binding Site, immunofixation grade) was coupled to CNBr-activated Sepharose beads (Pharmacia) at a concentration of 5 mg antibody/ml beads. A 250 μl aliquot of serum was mixed with 500 μl of antibody-coupled beads for 1.5 h at 25°C. Unbound proteins were removed by washing nine times with 25 mM Tris–HCl (pH 8.0), containing 140 mM NaCl, 1 mM $CaCl_2$, 0.5% (v/v) Nonidet P40, 0.1% (v/v) phenylmethylsulphonyl fluoride and these salts were finally removed by two rapid washes with distilled water. Bound Hp was eluted from the beads with 1 ml 0.1 M trifluoroacetic acid (TFA). The protein content in a 10 μl aliquot of the extract was determined by neutralizing it with 10 μl of 0.1 M NaOH, adding 10 μl 1 mg/ml BSA and 70 μl barbitone buffer, pH 8.6, and carrying out rocket electrophoresis [14]. The purity of the Hp preparations was checked by electrophoresis in 8% (w/v)

Table 1. Details of disease groups used in this study

Group	Age (range; median)	Tumour burden	Invasive growth	Detectable metastases	Chemotherapy
Ovarian cancer (progressive)	38–80; 66	high	7/7	7/7	7/7
Ovarian cancer (remission)	44–69; 50	low	7/7	0/7	7/7
Benign breast disease	22–60; 23	low	No	No	No
Early breast cancer	44–69; 57	low	5/7	3/7	1/7
Advanced breast cancer	32–71; 56	high	7/7	7/7	6/7

See text for further details.

polyacrylamide slab gels using a discontinuous Laemmli buffer system and the separated proteins were visualized by silver staining [2].

The monosaccharide composition of the purified Hp was measured using a high pressure anion-exchange chromatography system with a pulsed amperometric detector (Dionex carbohydrate system). The monosaccharides were released by mixing 800 μl of the Hp extract with an equal volume of 4 M TFA and hydrolysing for 5 h at 100°C. The TFA was removed by lyophilisation and the resultant material was reconstituted in 100 μl of distilled water. An aliquot of the latter was applied to a Dionex CarboPac PA1 column and eluted isocratically with 20 mM NaOH at a sensitivity setting of 1 K. Data was collected from the system using an Advanced Computer Interface and analysed using AI450 software. The monosaccharide composition was calculated by comparison with the elution profile of a mixture of monosaccharide standards (0.56 μg each of fucose, galactose, glucose, galactosamine, glucosamine and mannose). It should be noted that the fucose levels for the healthy and 'advanced' ovarian cancer groups in this study are higher than previously obtained [13]. This is because the previous analysis was done with a Hewlett-Packard integrator which was less sensitive than the currently employed AI450 software. The content of the other monosaccharides was unaffected by the method of analysis used. A separate portion of the Hp aliquot was hydrolysed in 0.1 M TFA for 1 h at 85°C to release *N*-acetyl neuraminic acid (NANA). An aliquot of this hydrolysate was applied to the PA1 column and eluted isocractically with a solution containing 50 mM sodium acetate and 100 mM NaOH at a sensitivity of 0.1 K. The NANA content was calculated by comparison with the elution profile of a 0.08 μg NANA standard. In pilot experiments, the NANA content of commercially available Hp

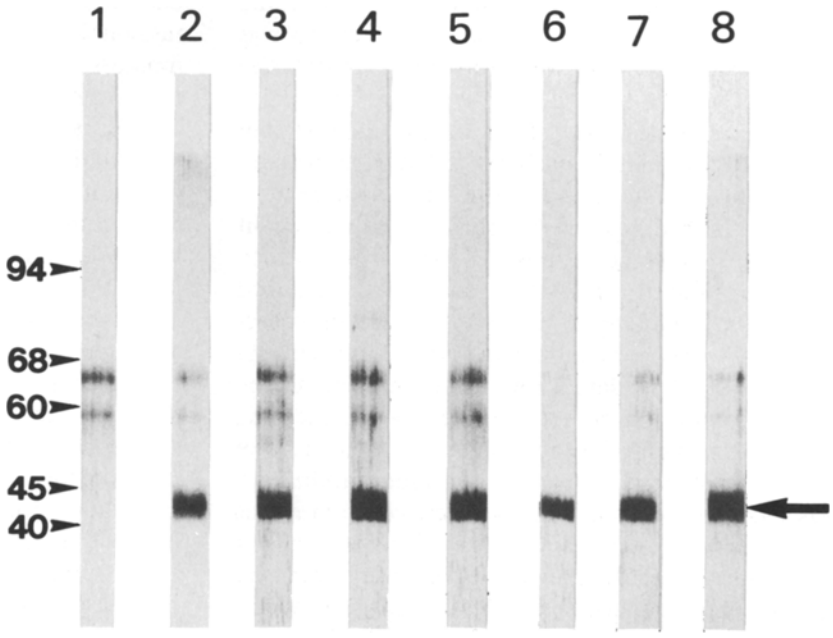

Figure 1. Silver-stained electrophoresis patterns (lanes 3–8) of haptoglobin purified from healthy women, progressive ovarian cancer, remission ovarian cancer, benign breast disease, early breast cancer, and advanced breast cancer sera, respectively. Lanes 1 and 2 contain reagent blank and commercial haptoglobin, respectively. The position of the haptoglobin (β chain) and the molecular weight markers (kDa) are indicated by an arrow and arrow-heads at the right- and left-hand sides of the pattern, respectively.

(Sigma, St Louis, MO) was shown to be unaffected by the extraction procedure.

Results

Figure 1 shows typical electrophoretic silver-stained patterns for Hp isolated from one serum sample for each group. Also shown are the patterns obtained when commercially-available Hp or antibody-coupled beads without added serum were subjected to the purification procedure. A single major band at 40–45 kDa was observed for all sera and the commercial Hp, with very minor bands at other positions. These latter bands, due to minor contaminants in the reagents used for electrophoresis, are present in all specimens and have been described by other workers [15]. The 40/45 kDa band is the β chain of Hp. The α chain is not seen because it runs in the solvent front of the 8% gel. The electrophoresis results show that the method used to isolate the Hp gave very pure Hp preparations.

Table 2 compares the monosaccharide composition of Hp from healthy women and women with ovarian cancer or breast cancer. Progressive ovarian cancer caused a large increase in the fucose content ($P < 0.0001$). Changes in the other monosaccharides were not significant ($P > 0.05$). When the ovarian cancer was in remission the fucose content fell and was not significantly different from normal ($P > 0.05$). The content of the other monosaccharides also fell in remission, but only the mannose and NANA were significantly lower ($P < 0.005$). If the results were calculated per three mannoses, the fucose concentration was still elevated in progressive disease ($P < 0.0001$) and fell in remission. However, there were also small but significant increases in the GlcNAc and galactose (Gal) concentrations that were maintained in remission (see Table 2 for P values).

Early breast cancer resulted in a significant increase in fucose content ($P = 0.019$), which further increased when the disease was advanced ($P = 0.005$). Changes in the other monosaccharides were not significant ($P > 0.05$). If the results were calculated per three mannoses, the fucose content increased as the disease became more extensive (see Table 2 for P values). The pattern of change for the other monosaccharides was more complicated. GlcNAc concentration was significantly higher in early breast cancer ($P < 0.05$), but this change was reversed when the disease was more advanced. N-acetylgalactosamine was not detected in any specimens, confirming the absence of O-glycosylation on this molecule [8].

Discussion

This study has shown that the fucose content of Hp is considerably increased in all groups where the women have progressive or advanced cancers. From our results, it seems that spread of the tumour is just as important as high tumour burden, because fucose levels were elevated in the early breast group. Although the tumour burden in this latter group was relatively low, invasive tumour was detected in 80% of the cases and metastasis was present in approximately 40% of the group.

In previous studies of cancer patients we showed that the amount of Hp that could be extracted from serum by the fucose-specific lectin, *Lotus tetragonolobus*, was related to tumour burden rather than metastases [3]. At first glance, it would seem that there is a discrepancy between these two sets of findings; however, further consideration shows that this may not be the case. The two studies may not be measuring the same thing. It has been shown that lotus binds to fucose that is present on oligosaccharides in a specific configuration [16]; therefore, the lectin-extracted molecules probably represent a subset of molecules. On the other hand, the current methodology gives the average fucose content per mole of protein for all the oligosaccharide side-chains on all the Hp molecules isolated.

Another reason for this discrepancy may be the difficulty in assessing the degree and extent of tumour involvement. It is impossible to separately monitor growing tumour, spreading tumour and total tumour burden in a precise way in human cancer, and the different conclusions from these two studies may be because they are investigating different aspects of the same situation. Nevertheless, from both studies one can conclude that the fucose content of Hp is low when the disease is benign or in remission and higher when the disease is progressive. Although ovarian cancer metastasizes frequently to the liver, it is thought unlikely that the changes in fucosylation are due to liver damage. This is because a previous study [3] showed that increases in the *Lotus* extractable Hp did not correlate with increases in the activity of aspartate aminotransferase, an enzyme that increases in liver damage.

The changes in the concentrations of other monosaccharides in cancer were minor by compari-

Table 2. Monosaccharide content of Hp from healthy women and women with ovarian or breast cancer

Group	Monosaccharide content								
	(mol/mol protein)					(mol/three mannoses)			
	Fuc	Man	GlcNAc	Gal	NeuAc	Fuc	GlcNAc	Gal	NeuAc
Healthy women	1.0 ± 0.48	19.4 ± 4.5	24.0 ± 4.2	14.4 ± 3.0	14.0 ± 1.4	0.16 ± 0.08	3.7 ± 0.2	2.3 ± 0.3	2.2 ± 0.4
Ovarian cancer (progressive)	3.7[a] ± 1.1	20.3 ± 4.4	29.0 ± 5.3	18.6 ± 4.7	13.6 ± 1.8	0.54[a] ± 0.14	4.3[b] ± 0.3	2.7[c] ± 0.3	2.1 ± 0.5
Ovarian cancer (remission)	0.59 ± 0.20	11.5[d] ± 3.6	18.7 ± 7.8	11.0 ± 4.2	10.3[d] ± 2.4	0.16 ± 0.06	4.7[e] ± 0.8	2.8[f] ± 0.3	2.5 ± 0.5
Benign breast disease	1.2 ± 0.59	19.6 ± 3.7	24.6 ± 4.8	16.2 ± 3.7	13.0 ± 3.6	0.18 ± 0.07	3.7 ± 0.3	2.4 ± 0.3	2.2 ± 1.1
Breast cancer (early)	2.2[g] ± 0.74	20.1 ± 5.6	28.5 ± 10.3	17.7 ± 6.1	15.3 ± 8.2	0.33[h] ± 0.10	4.3[i] ± 0.6	2.6 ± 0.2	2.3 ± 1.1
Breast cancer (advanced)	3.7[j] ± 0.96	23.6 ± 4.5	29.0 ± 5.5	17.8 ± 3.3	13.2 ± 1.2	0.50[k] ± 0.19	3.7 ± 0.4	2.3 ± 0.2	1.7 ± 0.4

Healthy vs progressive: [a] $P < 0.0001$; [b] $P < 0.0005$; [c] $P < 0.05$.
Healthy vs remission: [d] $P < 0.005$; [e] $P < 0.01$; [f] $P < 0.025$.
Early vs benign: [g] $P = 0.019$; [h] $P = 0.005$; [i] $P < 0.05$.
Early vs advanced: [j] $P = 0.005$; [k] $P = 0.05$.
All values are mean ± SD, Student's t-test was used for statistical analysis.
Only six values were available in the benign breast group for mol/mol Hp.

son with fucose. The elevated GlcNAc and Gal levels in ovarian cancer suggest that some glycan chains have an increased number of branches. However, this finding is independent of disease activity, which could indicate that it is due to some indirect cause, such as cytotoxic therapy or an inflammatory response. The general reduction in the glycosylation of Hp when ovarian cancer patients were in remission suggests that on some molecules, some glycosylation sites may be unoccupied; however, as mentioned above, when the results are expressed per three mannoses, the occupied glycosylated sites still appear to have increased branching. The results from the early breast group also suggest increased branching, but why this was not maintained in the advanced breast group is unclear. Other studies have reported variable findings for serum glycoprotein branching in cancer [see 11 for discussion].

The fucose content of Hp has been shown to be elevated in rheumatoid arthritis (RA) [17] and liver disease [18] as well as cancer. It could be argued, therefore, that this type of increase is closely linked to the well documented increase in Hp caused by an acute-phase reaction in these diseases. The circumstantial evidence, however, suggests that this explanation may be too simplistic. Firstly, the overall changes in the glycosylation in various diseases appear to be very different. The Hp extracted from RA sera by *Lotus* lectin gave sharp bands on electrophoresis, whereas that extracted from cancer sera under identical conditions was very diffuse with high molecular weight components [2, 7]. The magnitude of the change in fucose content of antibody-extracted Hp (residues/three mannoses) in these diseases was different: the increase being smallest for active hepatitis [18] and Crohn's disease (T. Goodarzi and G. A. Turner, unpublished observations) and largest for ovarian and breast cancer (this study). On the other hand, GlcNAc content was only slightly increased or unchanged in RA [17] or cancer whereas in Crohn's disease (T. Goodarzi and G. A. Turner, unpublished observations) and liver diseases [18] the change was larger. Secondly, the association between an elevated Hp level and changed glycosylation is very variable. In RA, increased fucosylation of Hp is strongly associated with an elevated Hp level [2]; and in other diseases such as bronchial pneumonia, ulcerative colitis or Crohn's disease, Hp can be massively elevated without any change in fucosylation [2, 7]. This conclusion agrees with the reported effects of cytokines on the synthesis of acute-phase proteins in cultured liver cells which suggest a partial uncoupling of the glycosylation and protein synthesis mechanisms [see 19 for discussion].

The effects of increased fucosylated Hp in cancer are unknown but it could be involved in the progression of the disease either by interfering with adhesive processes associated with immune reactions or with the adhesive processes occurring during tumour cell metastasis. As the fucosylated Le antigens appear to be involved in both mechanisms [20], circulating Hp and other serum proteins with elevated fucose [19] could inhibit this adhesion. Recent advances in the methods used for the isolation [2, 13, 21] and analysis [13, 22] of serum glycoproteins will hopefully answer some of these questions and determine if Hp has a role to play in the development of this disease.

Acknowledgements

We gratefully acknowledge the staff of Newcastle General Hospital, the Blood Transfusion Service and Mr R. Ng, Department of Clinical Biochemistry, for assistance in collecting the blood specimens, and the North East Cancer Research Campaign for financial support.

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(Received 20 January 1994; accepted in revised form 31 January 1994)