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Analysis of Glycoproteins Released from Benign and Malignant Human Breast: Changes in Size and Fucosylation with Malignancy

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Abstract—*Radiolabelled glycoproteins released into media from benign and malignant human breast tissue after* 48 h *organ culture have been analysed using SDS polyacrylamide gel electrophoresis and fluorography.*

Major differences were identified between benign and malignant tissues: (i) a considerably higher incidence of glycoproteins in the molecular weight range 210–280 kDa *detected from carcinomas than benign samples, with incidence being greater in poorly differentiated tumours; (ii) fucosylation of these glycoproteins released from carcinomas but not benign breast; (iii) heterogeneity, particularly of fucosylation, between the carcinomas but consistency amongst benign breast.*

A glycoprotein of MW 230 kDa *was of particular interest since it was not detected from any benign samples but was present in* 11 *of* 17 *carcinomas, when it was almost always fucosylated. This could prove to be a useful tumour marker.*

INTRODUCTION

THERE IS ample evidence that modifications in the glycosylation of cell surface glycoproteins and glycolipids are associated with malignant transformation of cells [1–3]. Such alterations could be of significance in determining tumour cell behaviour since cell surface glycoconjugates have been implicated in cell–cell and cell–substrate interactions [4]. Many of the studies which have examined glycoprotein structure and synthesis of human tumours have been confined to malignant cell lines [5–7], rather than primary neoplasms, and comparisons with normal cells has been limited.

The identification of glycosylation changes in human breast carcinomas could be of importance with regard to the recognition of tumour associated markers and in the prediction of tumour behaviour. Previous histochemical studies using lectins confirmed that there are differences in specific sugar groups between the glycoproteins of benign and malignant human breast and that some alterations are related to tumour differentiation or to some extent metastatic potential [8–11]. Heterogeneity in the *in vitro* utilization of radioactive labelled sugars by malignant human breast in comparison to normal has also been found by using tissue autoradiography [12]. However, these histological approaches cannot give information about differences in glycoprotein structure and size occurring with malignancy. In the present study we have analysed the nature of glycoproteins released by a range of benign and malignant breast lesions during *in vitro* labelling with radioactive labelled sugars, using SDS polyacrylamide gel electrophoresis and fluorography. The results have been related to tumour characteristics such as differentiation and metastasis.

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MATERIALS AND METHODS

Tissues

All breast tissues were obtained immediately after surgery. Approximately 1 g from each specimen was placed in 10 ml Eagles Minimal Essential Medium containing 200 U/ml penicillin and 200 μg/ml streptomycin and kept at 4°C until prepared for culture. Adjacent slices were fixed in 4% formaldehyde in saline and processed to paraffin wax. A total of six samples of benign proliferative disease, four fibroadenomas, 18 carcinomas and lymph node metastases for two of the carcinomas have been studied.

Reagents

Dulbecco's Modified Eagles medium with L-glutamine (DMEM), Eagles Minimal Essential medium (MEM) and penicillin streptomycin were from Gibco; organ culture dishes were from Falcon.

D-[6-^{3}H]Galactose (specific activity 31 Ci/mmol) and L-[6-^{3}H]fucose (specific activity 70 Ci/mmol) were obtained from Amersham International plc.

Electran grade acrylamide, *N,N'*-methylene-bis-acrylamide, sodium dodecyl sulphate, ammonium persulphate and *N,N,N',N'*-tetra-methylethylene-diamine (TEMED) were from BDH Chemicals Ltd. Molecular weight markers for SDS-PAGE (MWSDS-6H), riboflavin, phenylmethylsulphonyl fluoride (PMSF) and Coomassie Brilliant Blue R-250 (CBR-250) were obtained from Sigma Ltd.

Organ culture

Tissues were diced from 1 mm cubes using opposing sterile skin graft blades with dental wax as a cutting surface. The raft culture method [13] was employed, with tissues placed on defatted lens tissue supported by stainless steel grids at the gas/liquid interface of organ culture dishes. Tissues totalling 100–130 mg wet wt were used per dish with 1.5 ml of medium, all tests being performed in duplicate. DMEM with L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin was used throughout with no other additives. After a 2 h pre-incubation the medium was changed, and 20 μCi/ml of [^{3}H]-fucose or galactose added. For three samples of benign proliferative breast, two fibroadenomas and six carcinomas, incubations were in an atmosphere of 95% air/5% CO_2 at 37°C for 18 and 48 h. The remaining samples were incubated for 48 h only. The medium was collected and the protease inhibitor PMSF was added to each sample to a final concentration of 1 mmol/l prior to storage at −20°C until required. The cultured tissue was fixed and processed for assessment of viability.

Electrophoresis

Medium from each incubation was dialysed and concentrated. Total protein levels were assayed using the method of Bradford [14]. Samples were denatured for 3 min at 100°C in the presence of 5% SDS and 2% mercaptoethanol. For each specimen samples containing 100 μg total protein were applied to 10–20% linear gradient polyacrylamide slab gels and run using the discontinuous buffer system of Laemmli [15]. Comparisons of individual sugar incorporation by benign and malignant cultures were performed on the same gel, using both 7.5% and 10% polyacrylamide gels. Electrophoretic runs were calibrated with a high molecular weight standard mixture containing myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa). The gels were stained with CBR-250 and destained in a solution containing methanol/acetic acid/water (5:5:2 v/v). Calibration curves were plotted for each gel and the molecular weights of sample glycoproteins estimated by their relative mobilities.

Fluorography

After staining the gels were rinsed for 2 min in ultra pure water to remove excess acetic acid which would otherwise precipitate the fluor. They were then soaked in 300 ml of 1 M sodium salicylate for 30 min [16], immediately dried down and exposed to X-ray film (Fuji RX) for 14 and 21 days at −70°C. The resultant fluorograms were developed and scanned using a laser densitometer (LKB Ltd.).

Histology

Haematoxylin and eosin stained sections of the benign samples were assessed for the degree of proliferative change and of the carcinomas for type, using WHO criteria, and grade applying the Bloom and Richardson criteria with modification [17].

RESULTS

Comparisons of the incorporation of [^{3}H]sugars were made between (i) all benign specimens, (ii) carcinomas, (iii) benign and malignant samples and (iv) within individual cases. All comparisons were undertaken on samples analysed under the same percentage gel and electrophoresis conditions.

Comparison between 18 h *and* 48 h *cultures*

There was a similar distribution of glycoproteins identified from the 18 h and 48 h cultures for each of the samples tested (Fig. 1). The differences and similarities between the benign and malignant cases (see below) were evident after 18 h incubation.

There was no significant differences in the viability of tissues incubated for the two time periods, and the viability of all tissues incubated for 48 h was good, as assessed by morphology.

Comparison between benign tissues and carcinomas

Major differences were identified between benign and malignant specimens in glycoproteins (gp) of molecular weight greater than 200 kDa and in the incorporation of [^{3}H]fucose. Four gps in the MW range 210–280 kDa were identified in different combinations from all carcinomas whereas media from only four of the 10 benign samples contained gps within this range (Tables 1 and 2). One fibroadenoma released a glycoprotein of MW 210 kDa which showed weak incorporation of [^{3}H]-fucose and galactose (Fig. 2) but there was no evidence of [^{3}H]fucose incorporation into the higher molecular weight gps released from other benign tissues (Figs. 3 and 4). In comparison, there was a

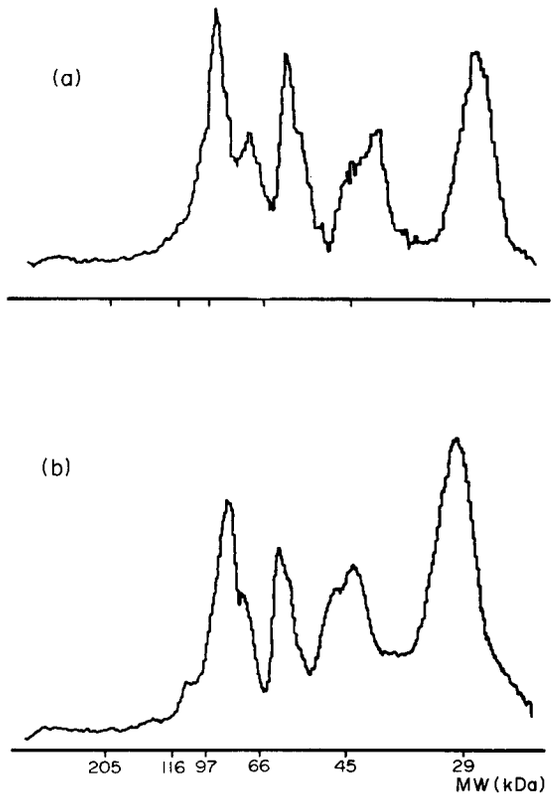

Fig. 1. A. Densitometric profiles of fluorograms of [^{3}H]fucose labelled glycoproteins released from benign hyperplastic breast. 10–20% *SDS-PAGE.* (*a*) 18 h *culture*; (*b*) 48 h *culture. B. Densitometric profiles of fluorograms of* [^{3}H]*fucose labelled glycoproteins released from a carcinoma.* 10–20% *SDS-PAGE.* (*a*) 48 h *culture*; (*b*) 18 h *culture.*

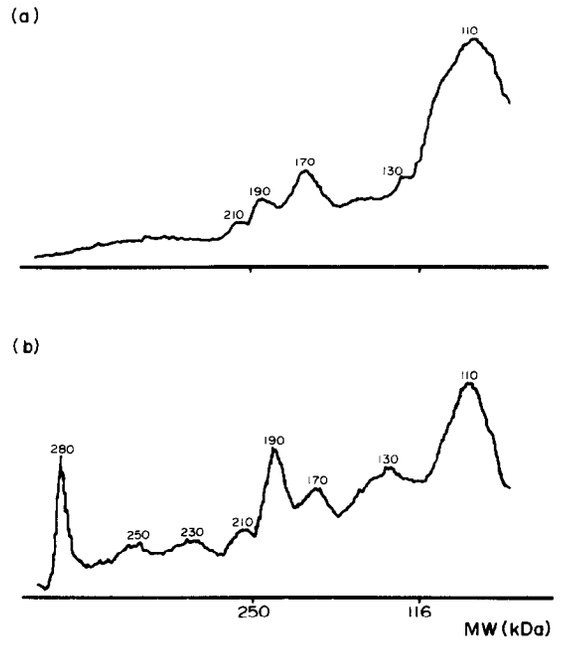

Fig. 2. Densitometric profiles of fluorograms of [^{3}H]*fucose labelled glycoproteins greater than* 100 kDa. 10% *SDS-PAGE.* (*a*) *Fibroadenoma* (*case* 3). (*b*) *Poorly differentiated carcinoma* (*case* 4).

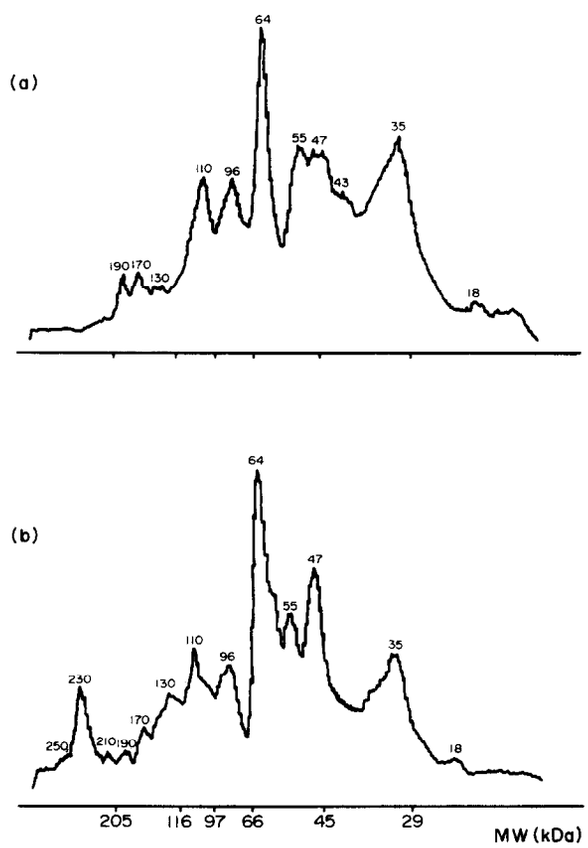

Fig. 3. Densitometric profiles of fluorograms of [^{3}H]*fucose labelled glycoproteins.* 10% *SDS-PAGE.* (*a*) *Benign hyperplastic breast* (*case* 9). (*b*) *Poorly differentiated carcinoma* (*case* 10).

Table 1. *The detection of glycoproteins* 210 kDa, 230 kDa, 250 kDa *and* 280 kDa *in media from cultured benign breast tissue*

Case No.	Histological features	Glycoprotein 210	230	250	280
1	Fibroadenoma	–	–	–	–
2	Fibroadenoma	–	–	–	–
3	Fibroadenoma	±FG	–	–	–
4	Fibroadenoma	–	–	–	–
5	Relatively normal	–	–	–	–
6	Focal mild hyperplasia	–	–	–	–
7	Diffuse mild hyperplasia	–	–	–	–
8	Diffuse mild hyperplasia	–	–	+G	+G
9	Focal moderate hyperplasia	+G	–	–	–
10	Diffuse moderate hyperplasia	+G	–	–	+G

– = absent; + = present; ± = weakly present; G = galactose; F = fucose.

Table 2. *The detection of glycoproteins* 210 kDa, 230 kDa, 250 kDa *and* 280 kDa *in media from cultured breast carcinomas*

Case No.	Type	Grade	Node	Glycoprotein 210	230	250	280
1	ID	2	pos	+FG	+F	–	–
2	ID	2	NK	+G	+F	–	–
3	ID	2	NK	–	–	+FG	–
4	ID	2	pos	+FG	+FG	–	+G
5	ID	2	NK	+F	+FG	–	–
6	ID	2	NK	–	+F	+FG	–
7	ID	2	pos	–	+F	–	–
8	ID	2	NK	+FG	–	+FG	+FG
9	ID	3	NK	+FG	+F	+FG	–
10	ID	3	pos	+FG	+FG	–	–
11	ID	3	pos	+G	+FG	+G	–
12	ID	3	NK	+FG	–	+G	+FG
13	ID	3	NK	+FG	–	+G	+FG
14	ID	3	pos	+FG	+F	–	+FG
15	ID	3	pos	+F	+G	–	+G
16	ID	3	neg	+FG	–	+FG	+F
17	Med	3	NK	+FG	–	+FG	+F
18	Secr	1	pos	ill defined high MW glycoproteins			

– = absent; + = present; G = galactose; F = fucose. ID = infiltrating duct carcinoma; Secr = secretory carcinoma; Med = medullary carcinoma. Node pos = metastasis present; Node neg = no evidence of metastasis; NK = node status not known. Grade 1 = well differentiated; 2 = moderately differentiated; 3 = poorly differentiated.

greater number of gps in the 210–280 kDa range identified in media from the carcinomas (Fig. 2) and these were frequently fucosylated (Figs. 2, 3 and 5, Table 2). The gp of MW 230 kDa was notable for not being detected from any of the benign samples but identified from 11 of 17 carcinomas, being fucosylated for 10 of these (Figs. 2, 3 and 5).

Differences were identified between the gps of less than 200 kDa from benign and malignant breast but these were generally quantitative or less consistent. The gp of MW 130 kDa was usually a more prominent band in the carcinoma samples (Figs. 2 and 3), whereas gp of 55 kDa was frequently more prominent in the benign cases (Figs. 3, 4–6). Several bands were readily identifiable for both benign and malignant specimens such as the gps of 110, 96, 64, 47, 35 and 18 kDa (Figs. 2–6).

Benign tissues

There was no significant difference in the number and MW size of the gps identified from the different cases, apart from the small number for which higher molecular weight gps were found. The latter appeared to relate to the degree of hyperplastic change (Table 1). Below 200kDa all gps identified showed incorporation of both [^{3}H]fucose and galactose (Fig. 4).

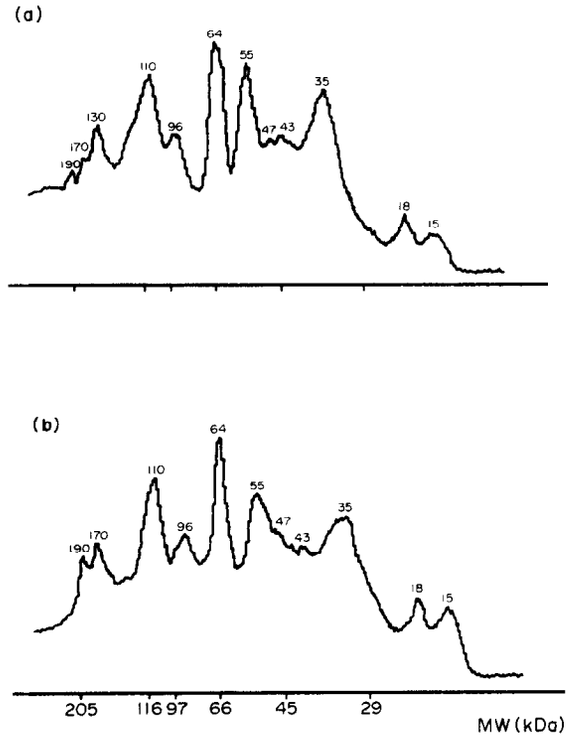

Fig. 4. Densitometric profiles of fluorograms of labelled glycoproteins released from benign mildly hyperplastic breast (case 7). 10–20% SDS-PAGE. (a) [^{3}H]Galactose label. (b) [^{3}H]Fucose label.

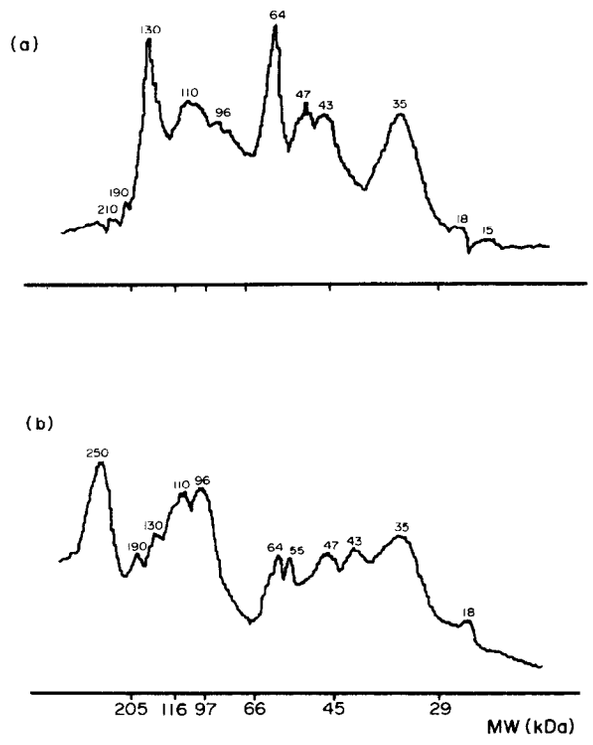

Fig. 6. Densitometric profile of fluorograms of [^{3}H]galactose labelled glycoproteins. (a) Fibroadenoma (case 3). (b) Moderately differentiated carcinoma (case 6).

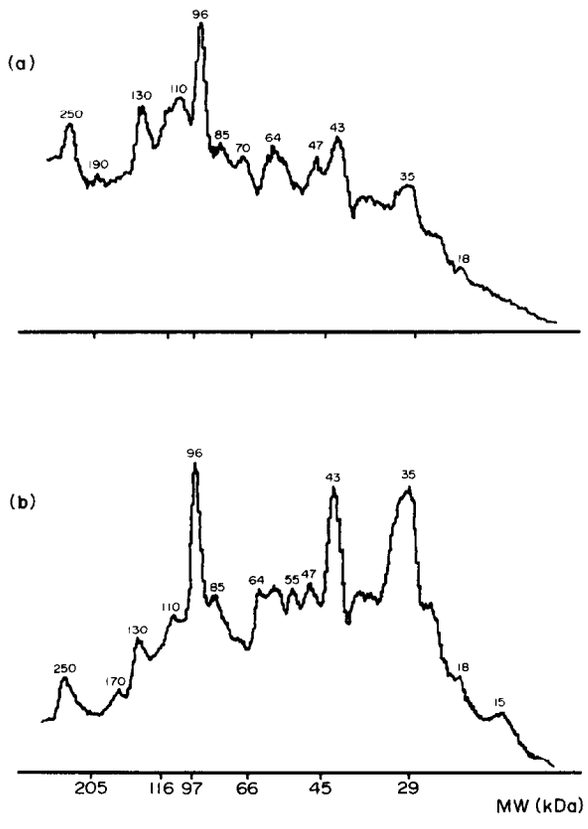

Fig. 5. Densitometric profile of fluorograms of labelled glycoproteins released from a moderately differentiated carcinoma (case 3). 10–20% SDS-PAGE. (a) [^{3}H]Galactose label. (b) [^{3}H]Fucose label.

Carcinomas

Adequate separation of the high molecular weight gps could not be achieved for one case, a secretory carcinoma. This was due to impaired migration from the stacking gel, which was probably related to the complex mixture of glycoproteins that are characteristic of this rare tumour.

An association was found between differentiation and the number of gps of MW 210–280 kDa identified, with 8/9 poorly differentiated carcinomas having three of the gps in comparison to 2/8 moderately differentiated tumours. Glycoproteins of 210, 250 and 280 kDa were more frequently identified from poorly differentiated carcinomas (gp210 9/9; gp250 6/9; gp280 6/9) than moderately differentiated tumours (gp210 5/8; gp250 3/8; gp280 2/8) whereas there was no difference for gp230. Insufficient information was available about lymph node status to draw any conclusions.

There was heterogeneity of fucosylation of the gps greater than 200 kDa (Table 2) and also in the MW region 40–80 kDa. An example of this is shown in Fig. 5 in which there are gps in this range which showed marked differences between the incorporation of [^{3}H]fucose and [^{3}H]galactose which was not seen in the benign samples (Fig. 4). However, there were carcinomas which behaved in the same way as the benign tissues.

Comparison of the lymph node metastases with their primary carcinomas showed that the patterns of gp release were similar, apart from heterogeneity of fucosylation of gps 50–55 kDa in one case. Histology confirmed that only metastatic carcinoma had been assessed.

DISCUSSION

This study has shown that there are significant differences in the nature of glycoproteins released from benign and malignant human breast *in vitro*, the main changes occurring with malignancy being related to glycoprotein size and fucosylation.

Increased expression of higher molecular weight glycoproteins occurring with malignancy has been reported for animal mammary tumours [18], including differences between rat mammary adenocarcinomas of low and high metastatic potential [19], but comparable studies on primary human tissues are limited. Gendler *et al.* [20] when examining the synthesis of [^{14}C]glucosamine-labelled glycoproteins by benign and malignant breast identified a group of acidic glycoproteins with molecular weights greater than 200 kDa which were more prominent in malignant specimens. In the present study there was a lower incidence of glycoproteins in the region 210–280 kDa from the benign tissues than the carcinomas. With the latter, the number of glycoproteins identified in this range increased with decreasing differentiation of the tumours, thereby stengthening the relationship of increased molecular weight size with malignancy.

Differences in lower molecular weight glycoproteins released from benign and malignant breast were also identified. Both increased (e.g. 130 kDa) and decreased expression (e.g. 55 kDa) of glycoproteins was found from tumours, whilst a number of bands were consistent between all samples. The band at 47 kDa could be the glycoprotein of MW 48 ± 6 kDa identified by Dermer and Tökes from benign [21] and malignant [22] breast, using similar methods. These findings indicate that the changes present in the higher molecular weight ranges were not related to technical variations. The similarities between the glycoproteins released from the 18 h and 48 h cultures for both benign and malignant tissues also suggest that the differences are not related to differences in cell surface turnover between the tissue types.

The higher (>200 kDa) molecular weight glycoproteins identified from benign and malignant breast also differed markedly in relation to the incorporation of [^{3}H]fucose. This was a feature of the glycoproteins released from carcinomas, although heterogeneity was prominent, but was essentially not found for the glycoproteins from benign breast. Heterogeneity of fucosylation of lower molecular weight glycoproteins (40–80 kDa) was also seen with carcinomas. Previous immunohistochemical studies have shown differences in expression of fucoproteins between benign and malignant breast, for example of the differentiation antigen 3-fucosyl-*N*-acetyllactosamine [23], which may reflect quantitative and/or qualitative differences in alpha-2- and alpha-3-fucosyl transferases or their regulation [24]. In studies of high- and low-metastatic cell lines [25–27] changes in fucose metabolism have been noted, which could have an influence on the biological behaviour of the cells.

The glycoprotein of MW 230 kDa was notable for being detected in 60% of carcinomas, when it was almost always fucosylated, but was not detected from any benign breasts. This was the only glycoprotein that showed such selectivity. Analysis by 2D electrophoresis, glycosidase digestion and peptide mapping is required to assess uniformity of structure between carcinomas. Similar methods of analysis need to be applied to the glycoproteins identified in this higher range, to assess similarities/differences between their protein and glycan structures, and also between those released from benign and malignant samples. Studies of those lower molecular weight glycoproteins which showed differences between benign tissues and carcinomas are also required. One of the major points of interest is the nature of the protein cores, since this would indicate whether the higher molecular weight glycoproteins released from carcinomas are formed completely *de novo* or whether they represent aberrant glycosylation of glycoproteins formed by normal breast. Data from analysis of mucins expressed by benign and malignant breast cells [28] indicates that the latter is more likely. Changes in the carbohydrate composition of glycoproteins involved in the function of normal cells could be of significance in determining the altered behaviour of malignant cells.

Glycoproteins synthesized by mammary cells of differing molecular weights have been recognized by many workers. A common approach has been by immunological analysis using monoclonal antibodies generated against breast tumours, cell lines and the milk fat globule membrane antigen. Of the higher molecular weight glycoproteins identified in this study, the one of 280 kDa may be the same or similar to that detected by the monoclonal antibodies generated by Major *et al.* [29]. Lower molecular weight glycoproteins recognized by antibody methods, e.g. of MW 43 kDa [30] and MW 19.5 kDa [31], may have been identified in the present study but did not exhibit the tumour specificity suggested by the previous reports.

As indicated, further work is required to investigate both the glycan and protein structure of these higher molecular weight glycoproteins, particularly gp 230 kDa. However it is anticipated that this will

provide an ideal immunogen for generating tumour-associated or even specific antibodies.

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