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Invited Review

The involvement of *Helix pomatia* lectin *(HPA)* binding N-acetylgalactosamine glycans in cancer progression

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Summary. The lectin from ***Helix*** pomatia, the Roman snail (HPA), recognises terminal alpha N-acetyl-galactosamine residues. A large number of lectin histochemical studies have demonstrated that expression of HPA-binding glycoproteins by cancer cells to be a marker of metastatic competence and poor prognosis in a range of common human adenocarcinomas, including those of breast, stomach, ovary, oesophagus, colorectum, thyroid and prostate. Around 80% of metastases arising from primary breast cancer are predictably HPA positive, but, intriguingly, around 20% do not express HPA binding glycoproteins reflecting the complexity of metastatic mechanisms and the further disruptions in cellular glycosylation that attend tumour progression. HPA binding is not an independent prognostic factor, but is strongly associated with the presence of metastases in local lymph nodes. It does appear to be independent of other clinical features of prognostic importance such as tumour size, histological grade, S-phase fraction, ploidy, and there is little convincing evidence of any association with oncogene expression or hormone receptor positivity. The precise nature of the metastasis-associated HPA binding partner(s) is a question of some interest, but thus far remains unclear. HPA will recognise, for example, the Tn epitope and blood group A antigen, but its prognostic significance appears to be through recognition of a much broader and heterogeneous array of N-galactosaminylated glycoproteins. Their synthesis appears to be mediated through alteration in expression or activity of one or more of the enzymes of glycosylation. The most likely putative roles of HPA-binding ligands in the metastatic cascade may be enhancement of invasive capacity, or interaction with an as yet unidentified lectin-like receptor facilitating adhesion processes. The prognostic information provided by HPA lectin histochemistry may be used clinically to inform the physician and aid treatment decisions; far more interesting is the challenge of further understanding the precise nature of the HPA-binding ligands, and defining their role in the complex mechanisms of metastasis.

Key words: Helix pomatia lectin, HPA, glycosylation, metastasis, GalNAc

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Introduction

It has been more than a decade since we first reported that expression of HPA binding glycans by breast cancer cells is associated with aggressive biological behaviour, metastasis, and poor long term patient prognosis (Leathem and Brooks, 1987a). Since that time, a number of studies from groups world-wide have confirmed the prognostic significance of HPA binding in breast cancer (Fenlon et al., 1987; Fukutomi et al., 1989, 1991; Alam et al., 1990; Noguchi et al., 1993a,b, 1994a; Thomas et al., 1993). Expression of HPA binding glycoproteins by cells of the primary tumour appears to be a powerful marker of poor patient prognosis. For example, in a 24 year retrospective study of 373 breast cancer patients, of which 293 (79%) had HPA positive tumours and 80 (21%) had HPA negative tumours, highly significant differences in survival were observed between the two groups. At 5 years after diagnosis, approximately 85% of patients with HPA negative tumours were alive compared with only approximately 55% of patients with HPA positive tumours; at 10 years after diagnosis, approximately 65% of patients with HPA negative tumours were alive compared with approximately 30% of patients with HPA positive tumours ($p<0.00001$) (Brooks and Leathem, 1991).

The lectin extracted from the albumin gland of ***Helix*** pomatia, the Roman or edible snail, has a nominal monosaccharide binding specificity for a-glycosidically linked N-acetylgalactosamine (GalNAc). Oligosaccharides containing GalNAc $\alpha 1 \rightarrow 3$ GalNAc non-reducing termini appear to be the best inhibitors of the agglutinin yet tested (Baker et al., 1983). The binding preference of the lectin can be summarised as follows:

GalNAc α1 →3 GalNAc β1→3 Gal α1→4 Gal ß1→4 Glc (Forssman specific pentasaccharide) > GalNAc α1→3 GalNAc > GalNAc α1→3 Gal ß1→3 GlcNAc > GalNAc (Baker et al., 1983; Wu et al., 1997).

The purified lectin has a molecular weight of 79 kDa. It consists of six identical polypeptide chains of 13kDa each containing a single carbohydrate combining site, and linked to form three 26kDa subunit dimers. (Hammarstrom and Kabat, 1971; Hammarstrom et al., 1972). At least 12 isolectins exist (Vretblad et al., 1979).

Mitchell and Schumacher (1999) have recently published an excellent review on HPA as a prognostic indicator and as a tool in cancer research in this journal.

Localisation of HPA binding to breast cancer cells at the light microscope and electron microscope level

At the light microscope level, HPA binding to breast cancer cells is generally observed in granular cytoplasmic pattern with marked cell surface localisation (Leathem and Brooks, 1987a,b; Brooks and Leathem, 1991) as illustrated in Figure 1. Ultrastructural studies have shown HPA binding localised at the cell surface of a number of breast cancer cell lines, including MCF7, BT549, BT20, T47D and HBL100, and in association with microvilli and submembranous granules in MCF7 cells (Mitchell et al., 1995). Recent confocal microscope investigations of permeabilised breast cancer cells, from a range of cultured breast cancer lines including HMT 3552, BT 474, MDA MB 435, MDA MB 468, ZR 751, MCF7, MDA MB 231 and DU 4475 have revealed intense Golgi labelling (unpublished results).

Menopausal status of patients

In our early report of the prognostic significance of HPA binding in breast cancer (Leathem and Brooks, 1987a), where a group of 179 patients was considered, we performed life table analysis on pre- and post-menopausal patients as separate cohorts. HPA binding was strongly predictive of poor prognosis in pre-menopausal patients but poorly predictive in post-menopausal women. In contrast, a later study by Fukutomi et al. (1989) reported no difference in the prognostic significance of HPA binding between pre- and post-menopausal women and, indeed, we also noted that in a larger study (Brooks and Leathem, 1991) of 373 cases, no difference in the prognostic significance of HPA binding in the two age cohorts (unpublished data). Fukutomi et al. (1989) suggest that the clearer survival differences in the pre-menopausal women may be due to there being a higher proportion of advanced carcinomas

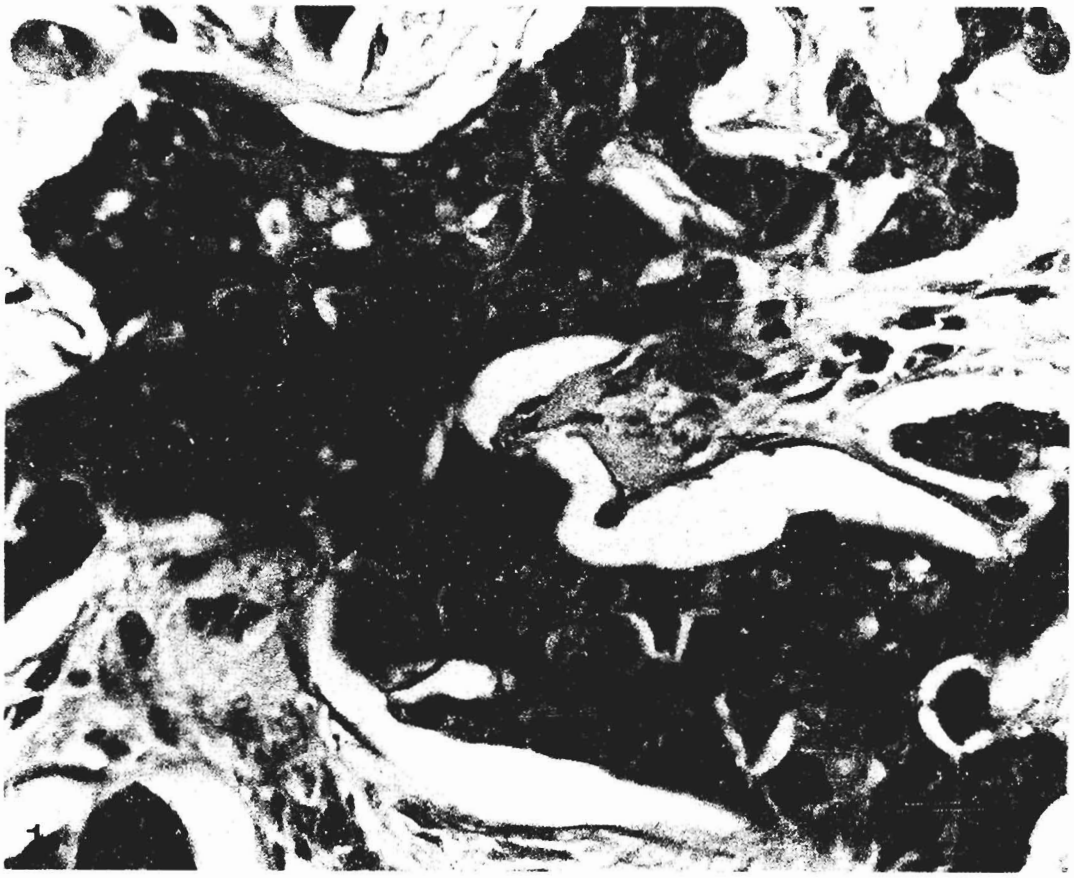

Fig. 1. HPA binding to primary breast cancer. Staining pattern is granular cytoplasmic with marked cell surface localisation. Bar: 30 μm.

in that group. We now believe that the apparent difference in the prognostic significance of HPA binding in the two groups was merely an artefact of small patient numbers in sub group analysis.

HPA binding to blood group sugars

HPA binding is not confined to metastatic cancer cells. The lectin will recognise any appropriate N-acetylgalactosaminylated structure. The blood group A sugar, for example, contains terminal N-acetyl-galactosamine (Fig. 2) and is recognised by the lectin. Thus, in blood group A or AB individuals binding is seen to endothelium and erythrocytes (Leathem and Brooks, 1987b; Fukutomi et al., 1991). The question arises as to whether the ABO blood group of breast cancer patients has any bearing on the HPA binding status of their tumours (Grundbacher, 1987), especially as an excess of blood group A individuals has been described in groups of breast cancer patients in Texas (Anderson and Hass, 1984) and in a population displaying a particularly aggressive form of the disease in Tunisia (Mourali et al., 1980). We have addressed this question and demonstrated that in 70 breast cancer patients in whom ABO blood group was known, no association between blood group and HPA binding to cancer was seen, although lectin binding to endothelium and erythrocytes was observed in group A and AB individuals (Leathem and Brooks, 1987b). Within individual cases, it is very apparent that no association is seen between positivity of endothelium/erythrocytes and positivity of cancer cells in the same cases, i.e. cancer cells are frequently positive for HPA binding while endothelium and red blood cells are negative, and vice versa (Brooks and Leathem, 1995; Ito et al., 1996a,b). This is illustrated in Figure 3. Comparison of staining patterns in tissue sections and probing of Western blots of extracted breast cancer glycoproteins using HPA in parallel with a monoclonal antibody directed against N-acetylgalactosamine (BRIC 66, King et al., 1991) confirm that, although HPA clearly does recognise and bind blood group A substance when it is expressed by breast cancer cells, the lectin recognises a much broader and heterogeneous selection of oligosaccharides (Brooks and Leathem, 1995).

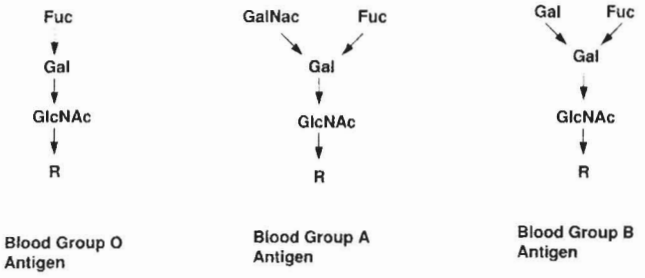

Fig. 2. The oligosaccharide termini of blood group A, B and O antigens. Blood group A contains terminal N-acetylgalacatosamine and is thus recognised by HPA. Fuc: fucose; Gal: galactose; GlcNac: N-acetyl-glucosamine; GalNac: N-acetylgalactosamine).

HPA binding to non malignant tissues

Alpha-N-acetylgalactosamine, the monosaccharide for which HPA shows greatest affinity, is only unusually seen in a terminal position on oligosaccharides of glycoproteins in normal human tissues. However, HPA binding to normal structures (other than erythrocytes and endothelium in blood group A/AB individuals, discussed previously) is sometimes observed. Of perhaps greatest interest is the observation that HPA frequently binds to epithelial cells, but never to myoepithelial cells, in normal breast. Binding is highly localised to the luminal surface of the epithelium in contrast to entire cell surface plus granular cytoplasmic localisation seen in cancer cells. This is illustrated in Figure 4. Strong staining of breast secretions is also sometimes observed (Leathem et al., 1983; Brooks, 1990; Mitchell and Schumacher, 1999).

We have also observed intense HPA staining of cells in a number of benign breast conditions, including hyperplasia, cysts and apocrine metasplasia (Brooks 1990). It remains unclear as to whether the glycoproteins recognised by HPA in normal and benign breast lesions are the same, or different, species to those expressed by malignant cells, and this is an area that warrants further investigation.

Expression of HPA binding glycans and prognosis of adenocarcinomas of sites other than breast

A number of studies have demonstrated a relationship between HPA binding and malignant potential or poor prognosis in adenocarcinomas at sites other than breast (reviewed by Mitchell and Schumacher, 1999). These include gastric carcinoma (Macartney, 1986; Kakeji et al., 1991, 1994; Maehara et al., 1995; Okuyama et al., 1998), ovarian papillary cystadeno-carcinoma transplanted into a SCID mouse model (Schumacher et al., 1996a), carcinoma of the oesophagus (Yoshida et al., 1993, 1994a,b), colorectal carcinoma (Ikeda et al., 1994; Schumacher et al., 1994a,b), cancer of the thyroid (Sasano et al., 1989) and prostate carcinoma (Shiraishi et al., 1992). This suggests, intriguingly, that the glycosylation change associated with metastatic competence and detected by HPA in breast cancer may be common to other human cancers arising from glandular tissues.

Expression of HPA binding glycans and prognosis of tumours other than adenocarcinomas

The prognostic significance of HPA binding in tumours arising from tissues of non-glandular origin remains less clear. It is interesting that HPA binding has been shown to be related to metastatic potential of cultured melanoma and sarcoma cells in an athymic

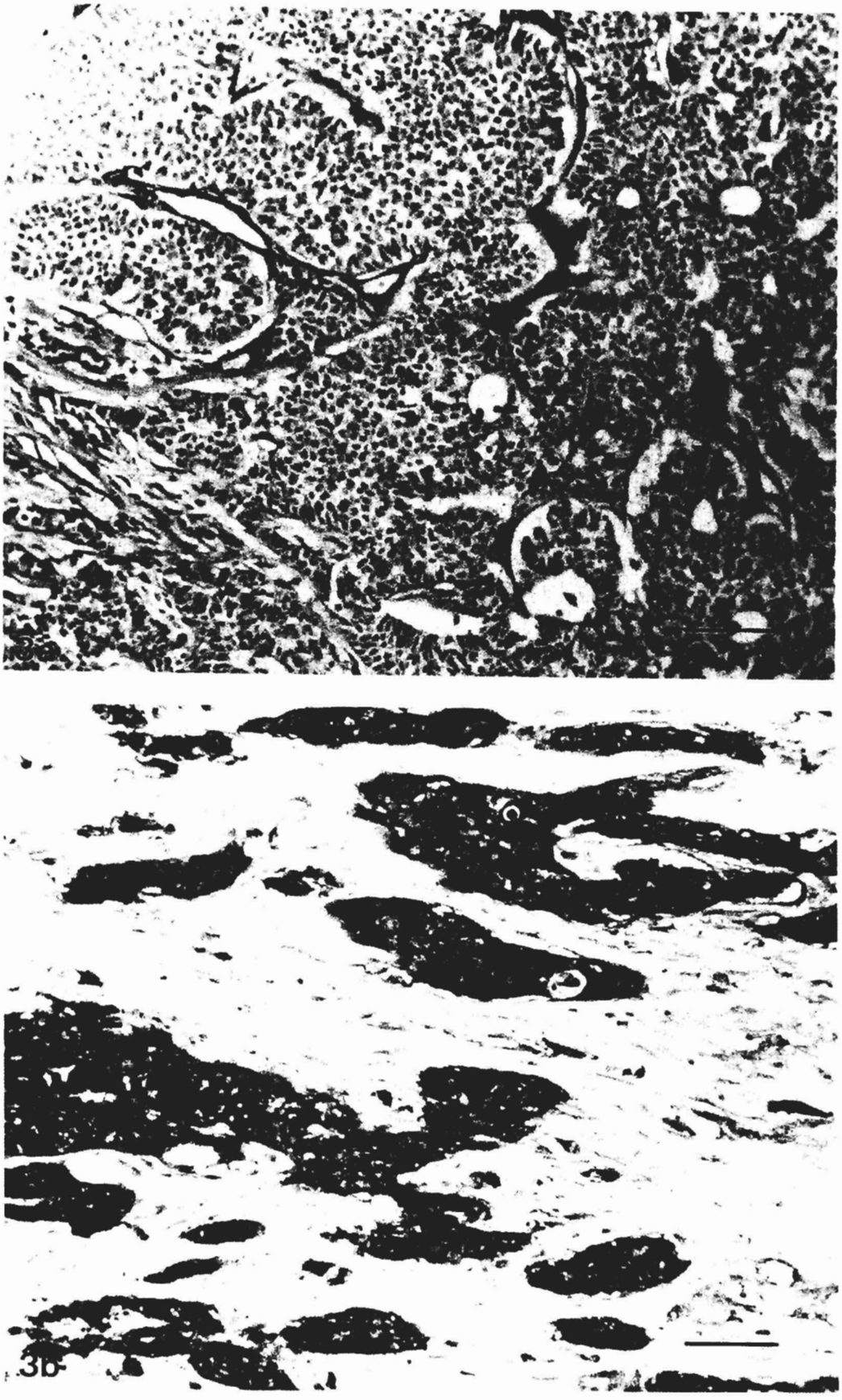

Fig. 3. There is no association between HPA binding to endothelium of blood vessels (indicating blood group A or AB individual) and binding to adjacent cancer cells. **a.** cancer cells HPA negative, endothelium of blood vessels HPA positive. **b.** cancer cells HPA positive, endothelium of blood vessels HPA negative. Bar: 80 μm.

nude mouse model (Kjonniksen et al., 1994; Rye et al., 1998), although studies have not yet been performed on human tumours.

HPA binding appears to be of little clinical significance in squamous cell tumours of the upper aerodigestive tract, where as many as 95% have been reported to be strongly positive (Schumacher et al., 1996c) giving little prognostic information. This may suggest fundamental differences in role of carbohydrate residues in metastasis between squamous cell carcinomas and in malignant tumours derived from glandular tissue.

We have recently found that juvenile and adult granulosa cell tumours and borderline tumours of the ovary do not express HPA binding glycoproteins at all, while, as might be expected from results obtained with adenocarcinomas arising from other sites, the small number of ovarian carcinomas examined did show variable levels of HPA binding (Fig. 5), as detailed in Table 1. In this study, the staining method described by us previously (Brooks and Leathem, 1991) was employed on paraffin sections of routinely formalin fixed and processed surgical specimens.

Table 1. HPA binding to human ovarian tumours.

TUMOUR TYPE	NUMBER OF CASES EXPRESSING HPA BINDING
Adult granulosa cell tumour	0/7
Borderline tumours	0/2
Juvenile granulosa cell tumour	0/2
Carcinoma	3/3

The importance of appropriate methodology for HPA cytochemistry

In spite of the fact that a large number of studies have demonstrated a clear relationship between HPA binding and poor prognosis in adenocarcinomas at various sites, in letters to The Lancet two groups have reported that they failed to do so (Galea et al., 1991; Taylor et al., 1991) and one of these studies has since been published in full (Gusterson et al., 1993). Galea et al. (1991) proposed that this discrepancy in findings may actually be due to differences in the immunocytochemical techniques employed: all of the studies that successfully illustrated a correlation between HPA binding and poor prognosis employed either unconjugated HPA or biotin labelled HPA, while the two studies that failed to do so used horseradish peroxidase-conjugated lectin. We (Brooks et al., 1996) stained a series of 373 primary breast cancers for the binding of HPA using both the direct method used by Gusterson et al. (1993) involving horseradish peroxidase-conjugated lectin and an indirect avidin-biotin technique employing

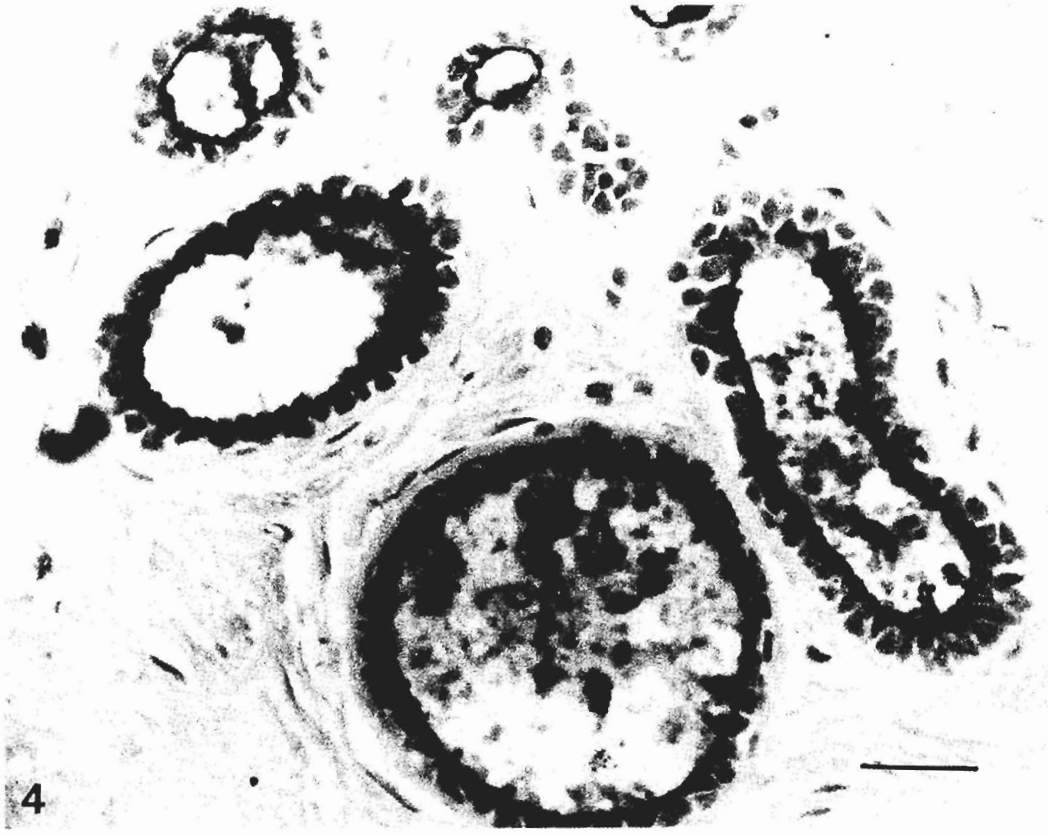

Fig. 4. HPA binding to normal breast ducts is localised at the luminal surface of the epithelial cells. Myoepithelial cells are negative. Breast secretions show some staining. Bar: 40 μm.

native, unconjugated lectin. The results were intriguing. Similar percentages of cancers stained positively with both methods (79% stained positive, 21% were negative in with both methods), but there was significant inconsistency in staining results when individual cases were examined. Approximately 10% of cases negative by the indirect method were positive by the direct (peroxidase-labelled lectin) method and vice versa. Life table analysis indicated that the indirect method yielded useful prognostic information, as reported in other studies - staining was associated with poor patient prognosis $p<0.0001$. The direct, peroxidase-labelled lectin method gave prognostic information of barely borderline significance $p<0.03$. The differences in results were not simply due to enhanced sensitivity of either method over the other, but to genuine large differences in binding characteristics of peroxidase labelled lectin. It seems likely that the conjugation of the lectin to the bulky horseradish peroxidase label molecule stearically interferes with the binding site of the lectin changing its binding characteristics. This was confirmed by peroxidase-labelled and native lectin binding to Western blots of breast cancer glycoproteins where the two forms of the lectin recognised a different array of molecules.

We would emphasise the importance of appropriate methodology - avoid use of horseradish peroxidase conjugated lectin when performing HPA histochemistry, and optimise the technique for the particular tissues, conditions of fixation and processing etc, as with any other lectin or immunohistochemical method. HPA binding results appear to vary with different conditions of fixation, processing and embedding (Schumacher et al., 1995).

Incorporation of appropriate positive and negative controls is also, naturally, of paramount importance (see Brooks et al., 1997a). The specificity of lectin binding can be demonstrated by its effective inhibition in the presence of 0.1M GalNAc. Staining should be abolished by omission of the lectin from the staining protocol. A case previously demonstrated to be strongly positive for HPA binding makes an appropriate positive control, as does normal human or animal (e.g. rat or mouse) kidney where the luminal surface of some, but not all, tubules should be strongly and selectively positive.

In our hands, virtually all adenocarcinomas are either obviously positive for HPA binding - i.e. all or most cancer cells staining or significant sub-populations of cancer cells staining - or obviously negative - i.e. few or no cells staining. We have previously defined criteria for designating cancers as either HPA positive or negative: cases are given a score for intensity of staining (negative: -, weakly positive: +-, positive staining: +, to extremely strong positive staining, ++++) and an approximate score for percentage of cancer cells positive

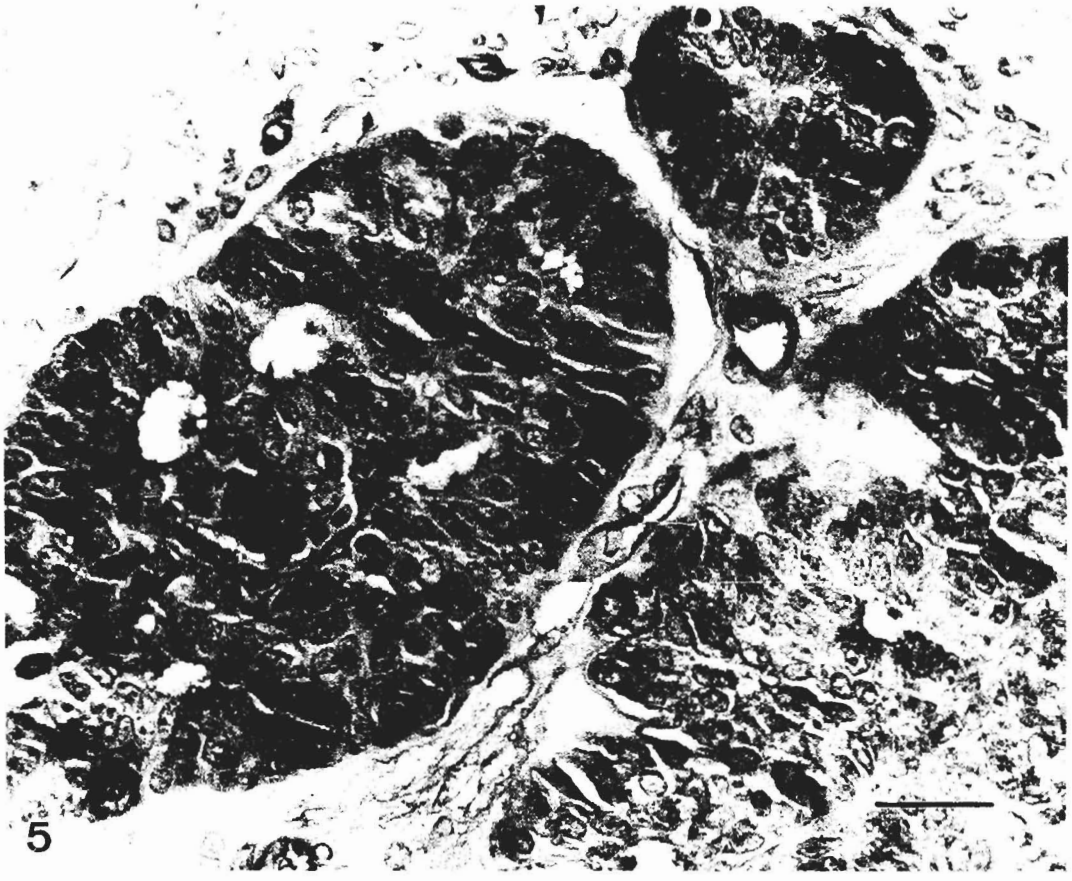

Fig. 5. Positive HPA binding to adenocarcinoma of the ovary. Bar: 40 μm.

(0 to 100%). Staining of normal structures such as breast ducts, endothelium or erythrocytes is ignored. Cases that receive a score of <5%+ or <50% +- are considered negative; cases that receive a score of ≥ 5% + or ≥ 50% +- are considered positive (Brooks and Leathem, 1991). This system has been adopted by other groups (e.g. Gusterson et al., 1993; Noguchi et al., 1993a,b; Thomas et al., 1993; Schumacher et al., 1994a; Noguchi et al., 1994a,b). Other workers have employed different criteria: Fenlon et al. (1987), for example, divided tumours into those where 0-50% of cells were staining positive vs. those where 51-100% of cells were staining positive. Studies by Fukutomi et al. (1989), Kakeji et al. (1991, 1994) and Maehara et al. (1995) also divided cases into a weak staining and a strong staining group - cases where 0-10% cancer cells stained were considered to be negative, cases where 11% or more cancer cells stained were considered to be positive. Yoshida et al. (1994a,b) and Shirai et al. (1996) simply classified cases as negative where no cells stained, positive where stained cancer cells were seen.

Binding to metastases

As expression of HPA binding glycoconjugates by primary tumours is associated with metastatic competence, the metastases themselves might also be expected to be invariably positive for HPA binding. The studies that have investigated HPA binding to metastases have clearly demonstrated that this is not necessarily the case.

Schumacher et al. (1992) investigated HPA binding to a series of breast cancer metastases to brain. The tumours had been fixed and processed immediately following surgical excision. 13/16 (81%) metastatic deposits were strongly positive for HPA binding while 3/16 (19%) were completely negative. We (Brooks and Leathem, 1989; Brooks and Leathem, 1999) have investigated HPA binding to breast cancer metastases to a variety of different sites including lymph node (n=10), liver (n=19), lung (n=15), bone/bone marrow (n=2), adrenal gland (n=6), skin (n=5), pleura (n=4), pituitary gland (n=4), uterus (n=3), and other sites (brain, thyroid gland, pancreas, spleen, contralateral breast, peritoneum kidney, gall bladder, ovary, meninges). The tissue samples were taken at autopsy. Once again, approximately 80% of metastases were strongly positive for HPA binding while approximately 20% were negative. There was no organ specificity to lectin binding - a similar proportion of metastases at all sites examined were positive/negative for HPA binding. Within any individual, metastatic deposits positive and negative for HPA binding were observed at different sites. Indeed, at single sites where multiple separate, discrete metastatic deposits were found - for example, several separate involved axillary lymph nodes - once again, a proportion of HPA-positive and HPA-negative deposits were observed. Fukutomi et al. (1989) examined the HPA binding characteristics of 63 involved lymph nodes from 113 primary breast cancers. In their study 46/63 (73%) of the lymph node metastases stained positive for HPA binding. In addition, 10 local recurrences from the same cohort of patients were investigated; 8/10 (80%) were HPA positive.

These findings appear curious: HPA positivity is strongly associated with the metastatic competence of the primary tumour, yet in all studies around 1/5 of metastases - at least some, presumably, arising from HPA-binding primaries - no longer express glycans recognised by the lectin. Heterogeneity of glycan expression is frequently observed in both primary cancers and their metastases - marked sub-populations of tumour cells are frequently noted - and it seems likely that aberrations in the machinery of glycosylation continue to occur throughout the natural history of the cancer. One explanation consistent with the observed HPA binding patterns of both primary and metastases, is that early in its natural history the primary tumour does not express HPA binding glycans; aberration in the machinery of glycosylation allow clones of HPA-binding cells to proliferate leading to, initially, HPA positive metastases; then, as further disruptions in glycosylation pathways occur during tumour progression, HPA binding is lost as new clones of cells, bearing further alterations in their glycan expression, emerge and become dominant. Consistent with this hypothesis are the results of Schumacher et al. who examined the HPA binding profiles of human ovarian papillary cystadenocarcinoma (Schumacher et al., 1996a) and human colon cancers (Schumacher et al., 1994b) transplanted into SCID mice and the metastatic tumours that subsequently developed in the animals. All primaries and metastases were HPA positive. As the animals were sacrificed at a relatively early stage after the establishment of metastatic tumour deposits it appears logical that the HPA binding characteristics of the primary tumours should be mirrored in their metastases. It would be interesting, although possibly unethical, to examine similar tumours left to develop for a longer period of time to determine whether, as hypothesised here, their glycosylation profiles would alter in response to further disruption in glycosylation pathways as a result of tumour progression. Given the biologically aggressive nature of HPA binding primary cancers, it is intriguing to speculate upon the natural history of HPA binding and non-binding metastatic tumour deposits at distant sites.

It would be consistent with observed results to suspect that HPA negative primary cancers, although generally a good prognostic group, are sometimes capable of metastasis. In all reported studies of human cancers, deaths from metastatic disease occur, albeit at a reduced rate, in patients with HPA negative primary cancers, and HPA negative cancer cell lines metastasise, again albeit at a reduced rate in comparison to HPA positive cell lines, in animal models (Schumacher and Adam, 1997). Metastases from these initially HPA negative primary cancers may also, during tumour progression, suffer disruption to the machinery of glycosylation leading to the emergence of HPA positive

clones.

In the study by Fukutomi et al. (1989), primary cancers and corresponding metastases to local sites were available in 4 cases. 2 primary cancers were HPA positive and 2 were HPA negative. One of the HPA positive primaries was associated with an HPA positive metastasis, the other with an HPA negative metastasis. One of the HPA negative primaries was associated with an HPA positive metastasis and the other with an HPA negative metastasis. Although assessment was only possible in such a tiny number of cases, the results confirm the complexity of the underlying mechanisms associated with the glycosylation changes in tumour progression.

Association with nodal status

The results of many studies consistently indicate that HPA binding is not an independent prognostic factor, but is strongly associated with the presence of metastases in local lymph nodes (Brooks and Leathem, 1991; Noguchi et al., 1993a,b; 1994a,b; Schumacher et al., 1994a; Maehara et al., 1995). This is a significant finding in that it implies that the expression of the HPA binding ligand(s) is associated with the physical ability of the tumour to metastasise to lymph nodes, and by implication to distant sites. It is widely accepted that that the histological presence or absence of axillary lymph node metastases is the single most informative prognostic indicator in patients with breast cancer (Adair et al., 1974). We have suggested (Brooks and Leathem, 1991) that HPA histochemistry on sections of primary tumour might provide valuable additional staging and prognostic information in patients receiving conservative surgery where lymph node status is not assessed.

Association with other clinical features

In studies where multivariate analysis has been carried out, it has been clearly demonstrated that HPA binding is not associated with other clinical prognostic factors such as tumour size, histological grade, S-phase fraction (Brooks et al., 1993), ploidy (Brooks et al., 1993; Noguchi et al., 1993a). However, as a marker of poor prognosis, HPA positivity is, unsurprisingly, seen in a larger proportion of tumours exhibiting other prognostically unfavourable characteristics such as high grade, large size, proliferating cell nuclear antigen labelling, aneuploid lesions, than in tumours with a more prognostically favourable clinical profile (Fukutomi et al.,1989; Brooks et al., 1993; Maehara et al., 1995).

Association with oncogene expression

Some groups have looked for association between HPA binding and over-expression of various proto-oncogenes by tumour cells. Noguchi et al. (1994a), for example, chose to investigate HPA binding and expression of nm23. The results were uninteresting in that they failed to demonstrate any prognostic value in nm23 expression, and it appeared unrelated to HPA binding. In this study, once again, HPA binding was a strong prognostic factor which multivariate analysis revealed to be strongly associated with lymph node status (axillary and internal mammary chain lymph nodes). Maehara et al. (1995) report that HPA binding is associated with over expression of p53 and higher growth potential in gastric cancers.

Fukutomi et al. (1991) investigated the relationship between HPA positivity and expression of three oncogenes - c-myc, int-2 and c-erbB2. Interestingly, they concluded that HPA binding was associated with gene amplification of the c-myc proto-oncogene, but found no relationship with either int-2 or c-erbB2. In contrast, Thomas et al. (1993) reported a strong association between HPA binding and over expression of c-erbB2 and suggested that stratifying patients on the basis of a combination of HPA binding and c-erbB2 expression might provide a powerful tool to discriminate sub-populations of patients with particularly bleak prognostic outlook who might benefit from more aggressive therapy. Noguchi et al. (1994b) found c-erbB2 expression to provide further prognostic information in both HPA binding and non-binding tumours - in both cases being associated with poorer prognosis. They suggest that a combination of HPA binding and c-erbB2 expression might provide more precise identification of sub-populations of patients at high risk of recurrence and short survival. Our group has assessed HPA binding and c-erbB-2 expression (using the monoclonal antibody 21N, a kind gift from Dr W. Gullick, ICRF Oncology Group, Royal Postgraduate Medical School, The Hammersmith Hospital, London) (unpublished results) and found no association between expression of the two markers.

This appears to be an area where there remains some confusion. There does not appear on balance to be strong support for an association between HPA binding and over expression of any proto oncogene believed to be of (poor) prognostic significance in human cancers. However, a possible relationship with c-erbB-2 expression is intriguing. Over expression of c-erbB-2 has been reported in a number of studies to be indicative of poor prognosis in a small proportion of breast cancer patients (Slamon et al., 1987; Berger et al., 1988; Slamon et al., 1989; Wright et al., 1989). It is perhaps possible that the relationship between HPA binding and over expression of this marker suggested by Thomas et al. (1993) might be an artefact of them both being poor prognostic markers, in much the same way as the majority of large tumours, or poorly differentiated tumours, are also HPA positive while multivariate analysis reveals that the factors actually act independently of each other.

Association with oestrogen and progesterone receptor expression

We have found no association between HPA binding by primary breast cancers and expression of either

oestrogen or progesterone receptors (unpublished results). Fukutomi et al. (1989) reported an association between HPA positivity and oestrogen receptor negativity. Again, it is possible that this merely reflects the likelihood of poor prognosis HPA positive tumours also sharing other poor prognostic features such as loss of expression of the oestrogen receptor.

Analysis of HPA binding partners

The precise nature of the metastasis-associated HPA binding partner(s) expressed by aggressive cancers is, clearly, a question of some interest, but thus far have been only incompletely characterised. The lectin, as already stated, has a nominal monosaccharide binding specificity for alpha-glycosidically linked N-acetylgalactosamine (GalNAc). Binding will also be inhibited, although to a lesser extent by N-acetyl-glucosamine (GlcNAc) (Hammarstrom and Kabat, 1969). It will thus recognise blood group A substance, Cad antigens, Forssman determinants (Baker et al., 1983), Tn epitope (Springer, 1989), as well as any glycoconjugate expressing glycans with terminal GalNAc or, possibly, GlcNAc moieties in normal or malignant cells.

Springer (1988, 1989) has suggested that the prognostic significance of HPA binding in cancers is owing to the recognition of Tn (GalNAcα-O-serine / threonine) epitope. Tn is not normally expressed in healthy tissues but is frequently over expressed in cancer cells and has been shown to be a marker of poor prognosis in a number of studies (e.g. Springer et al., 1975, 1985, 1986). Fukutomi et al. (1991) reported some co-expression of HPA and Tn detected immunohisto-chemically in breast cancers. We have shown, by immunohistochemistry of tissue sections and Western blotting of tumour homogenates, that although HPA does recognise Tn in cancer samples, it also recognises a much wider and heterogeneous profile of oligo-saccharides (Brooks and Leathem, 1995, Brooks and Leathem, 1999). Intriguingly, Rye et al. (1998) have recently reported that invasion of HPA positive LOX melanoma cells in a Matrigel assay could be effectively inhibited by prior incubation of the cells with HPA, but not with Tn, supporting the view that HPA-binding glycoconjugates other than blood group A trisaccharide or Tn may be important in GalNac-mediated metastatic mechanisms. They propose the Mac-2 (also known as Galectin-3) binding glycoprotein L3 (Koths et al., 1993) as an alternative candidate molecule. Galectin-3 or Mac-2 is an endogenous mammalian carbohydrate-binding protein that binds GalNAc, polylactosamine glycans, and ABH blood group sugars (Barondes et al., 1994; Hughes, 1994), increased expression of which has been correlated with increased metastatic potential (e.g. Mey et al., 1994). This is an intriguing idea that may warrant further investigation.

Forssman antigen is another possible candidate for HPA recognition (Wu and Sugii, 1988). However, Ito et al. (1996a) demonstrated that HPA staining was abolished by treatment with alpha-N-acetyl-galactosaminidase, but was insensitive to beta-N acetyl-hexosaminidase digestion making this possibility seem unlikely.

Mitchell et al. (1995) point out that in metastatic breast - and other - cancer there are a number of cell membrane constituents known to contain GalNAc and of possible functional importance in tumour progression and metastasis, including the epidermal growth factor receptor and the transferrin receptor. In Western blots of breast cancer cell line lysates separated by SDS-PAGE, they identified amongst a number of HPA-reactive bands, a prominent band running at approximately 90kDa. that they suggest could be related to the transferrin receptor. There remains, however, little evidence for this.

Since the prognostic significance of HPA binding has been demonstrated using formalin fixed, paraffin wax embedded tumour specimens, the carbohydrate residues of glycolipid molecules, although theoretically able to act as binding partners for HPA, can be excluded as being of interest since the alcohol/chloroform used in tissue processing would have eluted any glycolipids present (Schumacher et al., 1995). Hyaluronic acid, a GlcNAc-containing glycosaminoglycan appears to be of little interest as a binding partner for HPA (Schumacher et al., 1996b), and thus glycoprotein molecules appear to be the most likely HPA binding ligands of relevance to the metastatic process.

Initial analysis of HPA binding to glycoproteins isolated from primary breast cancers and their metastases reveals a heterogeneous profile of molecules consistent with the relatively broad binding specificity of the lectin (see Brooks, 1990; Brooks and Leathem, 1995; Streets et al., 1996). A heterogeneous array of HPA glycoproteins may also be derived from cultured breast cancer cell lines, human milk (Schumacher et al., 1995), and normal human tissues (Brooks, 1990; Streets et al.,1996). Detailed analysis of the oligosaccharides associated with such molecules is relatively complex and involves the use of specialised and expensive equipment. We have been developing techniques to extract and analyse the complex profiles of oligosaccharide structures released from both fresh, frozen and routinely formalin fixed and paraffin embedded whole tumour tissue (Leathem et al., 1995; Dwek et al., 1996; Brooks et al., 1998).

Ito et al. (1996a) have taken the approach of glycosidase digestion with lectin histochemistry to analyse the oligosaccharide structures involved in HPA binding to breast cancers. They conclude that the glycans are carried on a poly-N-acetyllactosamine containing a domain susceptible to endo-ß-galactosidase digestion. Their results also suggest that the HPA binding ligand(s) are bound to core protein by N-glycosidic linkage between N-acetyl-D-glucosamine and asparagine. Similar structures were also detectable in normal mammary epithelium suggesting that the ability

of carcinoma cells to produce poly-N-acetyllactosamine on N-glycans is preserved and only the peripheral portion of the chain is modified by, presumably, altered expression/activity of glycosyltransferases. O-glycosylation pathways appear to be much more severely disrupted in malignancy where backbone oligosaccharide structures are often completely lost to reveal core sialylated or unsialylated Tn or T antigen (Springer, 1984, 1989). Other studies have demonstrated dramatic differences in size and quantity of poly-N-acetyllactosamine moieties in carcinoma cell lines (Yamashita et al., 1984; Saitoh et al., 1992; Bierhuizen et al., 1994), colon cancer (Hakomori, 1989; Aoki et al., 1993) and thyroid cancer (Ito et al., 1995, 1996b; Yokota et al., 1995). There is evidence that these molecules are implicated in invasion and metastasis (Merkle and Cummings, 1987; Yousefi et al., 1991; Fukuda, 1994; Sawada et al., 1994).

Owing to the great heterogeneity of HPA binding glycoproteins in all human tissues, normal and malignant, it has proved difficult to identify a particular HPA-binding glycoprotein or glycoproteins expressed by aggressive cancers and not found in normal tissues that might be a putative metastasis-associated molecule. In fact the whole idea that a single molecule is responsible for the observed relationship between HPA binding and metastasis may be naïve. However, careful analysis of Western blots of SDS-PAGE separations of glycoproteins derived from primary breast cancers and their metastases, and normal control tissues has led to the identification of one such glycoprotein (Brooks, 1990; Streets et al., 1996). The molecule has a molecular weight of 55kDa. Purification and analysis revealed that it is actually an abnormally glycosylated form of IgA1 which is recognised by the lectin HPA. IgA1 derived from normal human tissues is not recognised by the lectin. The presence of this abnormally glycosylated immunoglobulin in tumour tissue is intriguing, and its functional role remains unknown. It seems unlikely, however, that this one HPA-binding glycoprotein is the key to the relationship between HPA binding and aggressive biological behaviour in tumours. The situation is likely to be considerably more complex. All evidence thus far is consistent with a general over expression of terminal GalNAc moieties on a complex and heterogeneous profile of proteins.

Mechanism of HPA binding ligand(s) expression

As stated previously, although the HPA binding partner(s) responsible for the association between HPA binding and metastatic potential are as yet poorly characterised, all evidence is consistent with there being a general over expression of terminal GalNAc moieties on the oligosaccharides associated with a wide range of cellular proteins, rather than there being a single, or a limited range of, HPA binding glycoproteins of interest. The most plausible explanation for such a general over-expression of N-acetylgalactosylated oligosaccharides would be an alteration in the expression or activity of one or more of the enzymes of glycosylation. Schumacher et al. (1995) have reported that normal breast epithelium expresses a discrete and limited repertoire of N-acetylgalactosylated glycoproteins, as detected by HPA binding to Western blots, in contrast to breast cancer samples which show significantly more heterogeneous expression. This observation is entirely consistent with an aberration in glycosylation pathways leading to general increased expression of terminal GalNAc moieties. Defects in glysosylation pathways leading to over-expression or under-expression of certain glycans have been repeatedly described to be associated with transformation to malignancy and to accompany cancer progression and evolution of the metastatic phenotype (e.g. Dennis et al., 1987; Yagel et al., 1989; Yang et al., 1994; Brockhausen et al., 1995; Nakano et al., 1996; Pierce et al., 1997).

In normal tissues, terminal GalNAc is relatively unusual, commonly being masked by sialic acid. Increased expression of terminal GalNAc may therefore be a result of either up-regulation or abnormal function of an GalNac transferase, or down regulation or inactivity of a sialyl transferase. The latter is highly plausible as numerous studies have reported disruption in sialylation in disease and malignancy (e.g. Sen et al., 1994; Vierbuchen, 1995; Yamashita et al., 1995). Intriguingly, however, Yuyama et al. (1995) have recently reported over-expression of a ß-1,4 GalNAc transferase gene in gastric and colon cancer in comparison to adjacent normal mucosa. Zhang et al. (1997), in an extensive analysis of more than 45,000 different genes in normal colonic mucosa and colon carcinoma, found surprisingly few differences, but reported down regulation of a sialyl transferase gene.

This is undoubtedly an area that warrants further investigation and research is currently underway in our laboratory to pinpoint the alterations in the normal glycosylation pathways that are responsible for the over expression of HPA binding partners.

Lectin binding to complex oligosaccharides and GalNAc-binding lectins other than HPA

There are numerous GalNAc-binding lectins available commercially with nominally similar sugar binding specificity to HPA. For example, *Dolichos biflorus* lectin, *Bandeiraea simplicifolia* isolectin I-A4, *Caragana arborescens* lectin, *Vicia villosa* isolectins, *Wisteria floribunda* lectin, and many others. It might be supposed that these lectins would give similar results to HPA and provide alternative prognostic markers; but in fact this is not necessarily the case. We have demonstrated that in tissue histochemistry lectins with identical nominal sugar binding specificity actually give very different staining results (Brooks et al., 1994; Khan et al., 1994; Brooks and Leathem, 1999). The reason for this is that lectins with identical nominal sugar binding specificity, for example those said to be specific for α-D-

GalNAc (for example *Helix pomatia* lectin HPA, *Helix aspersa* lectin HAA, *Dolichos biflorus* lectin and so on) may actually have very different, as yet uncharacterised, complex natural binding partners and thus yield very different results.

Lectin binding specificity is commonly quoted in terms of the monosaccharide (or sometimes di- or tri-saccharide) that most effectively inhibits lectin binding - so, for example, HPA is said to be specific for α-D-GalNAc, Concanavalin A for α-D-mannose, *Ulex europaeus* isolectin I for α-L-fucose etc. However, this is an oversimplification of the way in which lectins recognise their natural binding ligands, which are largely uncharacterised, and generally are complex oligosaccharide structures rather than simply mono- or di-saccharides. Lectins will bind with much greater avidity to a complex carbohydrate structure resembling their natural binding ligand (for example, oligosaccharides expressed as part of glycoproteins in a tissue section) than to a simple mono- or di-saccharide. The nature of lectin binding to its natural binding partner is complex, and involves specific recognition of terminal and subterminal sugars, and in three dimensions. The binding site may require the action of ions such as calcium or magnesium and may encompass part of the protein backbone as well as the oligosaccharide structure itself (Brooks et al., 1997b). The binding partners of most lectins remain imprecisely characterised and lectins considered 'identical' in terms of monosaccharide specificity, possess the ability to recognise fine differences in more complex structures (Debray et al., 1981). Thus it is unreasonable to expect all lectins with similar nominal sugar binding to give identical results in histochemistry. In our experience, the only lectin that gives similar results to HPA is the lectin from *Helix aspersa*, the garden snail HAA (Kahn et al., 1994). This is perhaps unsurprising as the species are closely related. We have recently reported very different binding patterns of HPA and DBA to a series of breast cancer metastases to different sites (Brooks and Leathem, 1999). Laferte et al. (1995) report that although DBA affinity chromatography resulted in near quantitative recovery of glycoproteins detected by their monoclonal antibody 3A7 (which recognises a determinant on type 2 chain blood group A and B oligosaccharides) from detergent lysates of normal rat colon, immunoreactive glycoproteins from colon cancers or HT29 colon cancer cell line bound only poorly to DBA, but could be recovered by HPA affinity chromatography.

The involvement of HPA binding ligands in cancer metastasis

There is therefore extremely strong evidence that an alteration in glycosylation pathways leading to an over-expression of N-acetylgalactosylated glycoproteins attends increased metastatic potential and thus poor prognosis in adenocarcinomas from a range of sites. The identification of HPA binding as a prognostic marker is in itself interesting and, potentially, clinically useful, but far more fascinating is the determination of a role for HPA-binding partners in the actual mechanisms of the metastatic cascade.

A number of studies report increased metastatic potential of HPA positive, in comparison with HPA negative, cell lines in animal models of metastasis. Schumacher and Adam (1997), for example, report successful experimental metastasis in 23/26 severe combined immunodeficient (SCID) mice innoculated with HPA positive colon (HT29) or breast cancer cell lines (MCF-7, T47D) compared to only 2/38 innoculated with HPA negative colon (COLO320DM, SW480, SW620) or breast (BT20, HS578T, HBL100) cell lines. The two animals developing metastases after innoculation with HPA negative cells had both received colon cancer cells of the line CACO2; 6 other animals treated in the same way did not develop metastatic disease. Rye et al. (1998) describe how LOX, an HPA positive melanoma cell line, showed an invasive potential more than 6 times that of FEMX-I, an HPA negative melanoma cell line, in a Matrigel invasion assay. Intriguingly, invasion of LOX cells was effectively inhibited in the presence of HPA, but not by Tn epitope.

There is increasing interest in the involvement of complex sugars and their specific receptors (lectins, galectins, selectins, etc.) in a whole range of biological phenomena in health and disease. Glycobiology is at present an area of intense research activity. Changes in cell surface glycoproteins are generally thought to be associated with altered cell adhesion properties of cells and with the development of invasive and metastatic phenotype (Dennis et al., 1987; Dennis and Laferte, 1989; Cornil et al., 1990; Sell, 1990; Banerjee et al., 1995). Although it is, of course, possible that the relationship between the increased expression of GalNAc sugars and metastastatic competence may be coincidental, it seems highly likely that the sugars are actually implicated in the metastatic cascade. Metastasis is an extraordinarily complex, multistep process that is as yet incompletely understood. The most likely putative role of GalNAc glycans in this process might be their interaction with an as yet unidentified receptor (perhaps an endogenous lectin, selectin or galectin) leading to cell adhesion or signalling events. This type of phenomena has already been demonstrated for other glycans, for example, the binding of tumour cell surface ß1-4 linked galactose to lectin(s) on endothelial cells (Cornil et al., 1990). Ongoing research in our laboratory is aimed at investigation of the role of GalNAc glycans in cell adhesion events and in identification of a putative receptor/lectin.

Implications for therapy and future directions

HPA lectin histochemistry applied to routinely formalin fixed and processed paraffin wax sections of adenocarcinomas from a number of common sites

(breast, colon, stomach, oesophagus, prostate) provides prognostic information that can be used clinically to inform the physician and aid treatment decisions. Histochemistry is a standard and established technique in most treatment centres and cost implications are negligible. This approach may be of particular relevance in breast cancer where conservative surgery is commonly performed in preference to the more mutilating mastectomy. Here, to minimise the surgical assault on the patient, local lymph nodes (in the axilla and internal mammary chain) are often inadequately sampled or not taken at all, and the valuable prognostic information they carry is lost. As HPA binding to detect altered glycosylation in cells of the primary tumour appears to be not only an excellent prognostic marker in its own right but also strongly linked to nodal status, HPA binding to routinely prepared paraffin sections of the primary tumour may provide additional staging information to aid treatment decisions in patients whose nodal status remains unknown (Brooks and Leathem, 1991). This point was highlighted by an editorial in the Lancet at the time that our original results were published ('Talking Points' in The Lancet of July 13, 1991). Several authors have suggested that HPA histochemistry in conjunction with other biochemical markers such as c-erbB2 (Noguchi et al., 1994b), p53 (Maehara et al., 1995), analysis of DNA ploidy (Noguchi et al., 1993a), or carcinoembryonic antigen (CEA) expression (Fukutomi et al., 1989) may provide a strong profile of prognostic information in breast cancer patients. Kakeji et al. (1994) recommended that as HPA binding was associated with lymphatic invasion, metastasis and poor prognosis in gastric cancer, careful follow up and aggressive therapy would be appropriate for HPA positive patients. HPA positivity might also be indicative of a sub-set of lymph node negative patients that would benefit from aggressive adjuvant chemotherapy owing to the likelihood of occult disease.

Okuyama et al. (1998) have recently reported that gastric carcinoma patients with HPA positive tumours where there is an unusually strong dendritic cell infiltration enjoy a significantly better prognosis than those whose HPA positive tumours have only slight dendritic cell infiltration. Altered glycosylation, as detected by HPA positivity, may increase immunogenicity of HPA positive cells and stimulate immunological host surveillance in these cases. Okuyama et al. (1998) suggest that boosting dendritic cell infiltrate by administration of immunochemotherapy containing the biologic response modifier OK-432 may have beneficial effects for patients with HPA positive tumours and slight dendritic cell infiltrate.

In addition to its application as a prognostic/staging marker to aid clinical treatment decisions, the association of HPA binding with metastatic competence raises a number of questions regarding new approaches to our understanding of the mechanisms underlying the metastatic cascade, diagnosis and therapy. In vitro and in vivo models of invasion and metastasis provide the means for functional investigations into the role of N-acetylgalactosaminylated glycans in cancer progression (see Mitchell and Schumacher (1999) for review).

As yet, the oligosaccharide binding partner of HPA is far too incompletely characterised to assess whether it may carry potential as a target for synthetic anti-carbohydrate vaccines, but promising research using related tumour associated carbohydrates such as the Tn (GalNAc-α-1-O-serine/threonine), T or Thomsen-Friedenreich (Gal-ß1-3 GalNAc-α1-O-serine/threonine) and sialosyl Tn (NeuAc α2-6 GalNac α-O-serine / threonine) antigens is underway (see Toyokuni and Singhal, 1995). Such vaccines have proven effective in blocking challenge by highly invasive mouse mammary carcinoma cells in a murine model (Singhal et al., 1991) and may be of use in preventing recurrence of human breast cancer (Springer et al., 1993). Research in our group is aimed at examining the immune response of patients to tumour derived GalNAc glycans; it is possible that boosting of the patient's immune response to tumour carbohydrate antigens may be a novel approach to disease control and therapy. Alternatively, specific monoclonal antibodies directed against tumour associated carbohydrates may eventually be of value in imaging occult metastases or in directing pro-drug therapy.

HPA binding to its, as yet incompletely characterised, binding partner provides useful prognostic information in human adenocarcinomas at a number of different sites. This in itself is an interesting and potentially clinically useful finding. The future challenge is to further characterise the tumour associated oligosaccharide binding partner(s) of HPA and to define their role in the progress of the disease. Elucidating the molecular mechanisms underlying their synthesis also remains a challenge. The 'how?' and 'why?' of N-acetylgalactosaminylated, and other, glycans in the mechanisms of tumour metastasis promise to be fruitful directions as far as our understanding of, and intervention in, tumour progression is concerned.

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