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Invited Review

The use of the lectin *Helix pomatia* agglutinin (HPA) as a prognostic indicator and as a tool in cancer research

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Summary. Progress in treatment for cancer has enabled extension of the disease-free interval, and of the quality of life for patients, but there has been very little improvement in overall survival rates. The main reason for this has been the ineffectiveness of current therapies to kill all the cancer cells once they have spread to distant sites to form metastatic deposits. One marker which has proved to be useful in identifying those cancers which have the potential to spread is the lectin *Helix pomatia* agglutinin (HPA). In clinical studies, HPA binding to primary tumours in tissue sections has been of prognostic value in breast, colon and gastric cancer, while no prognostic significance for HPA could be detected in tumours of the head and neck. These studies hence indicate that HPA is best suited to recognise a glycotope on adenocarcinomas. In several studies, HPA reactivity is equal or superior to other classical markers of metastatic potential. Since HPA is a marker of prognosis at the level of individual tumour cells, human tumour cell lines were screened for their HPA positivity. When transplanted into severe combined immunodeficient (scid) mice, HPA positive human breast and colon cancer cells metastasised while HPA negative cancer cell lines in general did not. In order to define HPA binding glycotopes at the molecular level, isolated cell membrane glycoproteins were exposed to labelled HPA on nitrocellulose membranes after Western blotting procedure. The majority of the isolated cell membrane glycoproteins bound HPA indicating that not a single HPA binding glycoprotein exists, which is associated with the metastatic phenotype. Functional investigations using the human/scid mouse chimeras will aid in the identification of those HPA positive glycoproteins which are functionally involved in the metastatic cascade.

Key words: *Helix pomatia* agglutinin, Cancer, Metastasis

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Introduction

It is alarming to note that there has been no recent substantial improvement in survival rates amongst cancer patients suffering from common forms of cancer (Sporn, 1996), despite improvements in the understanding of the molecular basis of cancer (Bailar and Gornik, 1997). The major reason for this is that there is still no effective therapy once the cancer has spread. Notwithstanding the increasing armamentarium of chemotherapeutic tools at the disposal of the oncologist it is not possible to kill what have become known as immortalised metastatic cells which are resistant to conventional drugs that interfere, for example, with cell division. Moreover, mortality rates have remained sadly stable in spite of increased detection and prevention rates, because of an increasing incidence of cancer.

The process of metastasis formation is a multistep phenomenon, often referred to as the metastatic cascade, (Hart et al., 1989; Hart and Saini, 1992), involving the loosening of tumour cells from the primary tumour, invasion of blood/lymphatic vessels, survival of the cells in the circulation, migration out of the vessels, and subsequent growth at the distant site. Since all these steps have to be carried out sequentially it has proved impossible to identify the rate limiting step of metastasis formation.

Carbohydrates and cancer

Many of the interactions between tumour cell/host cell and tumour cell/extracellular matrix described above are mediated by carbohydrate residues of the glycocalyx of the tumour cell surface, and those of the extracellular matrix. These carbohydrate residues of the glycoconjugates are readily identifiable by sugar-binding proteins of non-immunological origin which are known as lectins. The latter are found widely distributed in nature, ranging from plants, invertebrates, and to mammals including humans (Lis and Sharon, 1986). The word lectin means to choose and was first coined by Boyd (1963), though the ability of lectins to agglutinate

red blood cells was first reported by Stillmark (1888), and is a property relating to the expression of carbohydrate molecules on the red cell membrane. Indeed, some lectins are specific for some human blood groups and have, therefore, been widely used in blood group typing, whilst others are mitogenic and have, therefore, been used as growth stimulators in cell culture systems. Lectins have also been used in cancer research because it has been shown that some lectins specifically bind to particular cancer cells (Aub et al., 1963, 1965; Burger and Goldberg, 1967; Burger, 1969).

The lectin from the Roman snail *Helix pomatia* agglutinin (HPA) has a main carbohydrate specificity for N-Acetyl-Galactosamine (GalNAc), and was first isolated from the albumin gland of the Roman snail *Helix pomatia* by Hammarström and Kabat (1969). It was found to be a protein which specifically agglutinated human blood group A erythrocytes, of molecular weight 79 000, and contains about 7% carbohydrate side chains. HPA has been shown to bind to cancer cells of some tumours which have metastatic potential, and is correlated with the poor survival chances of these patients. This article will review the data which have been obtained using this lectin in the prediction of prognosis in a variety of different cancers (Table 1).

Table 1. Use of HPA as a prognostic indicator in different cancers in clinical studies and animal models.

CANCER	REFERENCE	ANIMAL MODEL
Breast carcinoma	Leathem et al., 1983, 1984, 1985 Leathem and Brooks, 1987 Brooks and Leathem, 1991 Fenlon et al., 1987 Fukutomi et al., 1989 1991 Thomas et al., 1993 Alam et al., 1990 Brooks et al., 1996 Noguchi et al., 1993	
Colorectal	Ikeda et al., 1994 Schumacher et al., 1994a,b	
Oesophageal	Yoshida et al., 1993 Takahashi et al., 1994	
Gastric	Kakeji et al., 1991, 1994	
Prostatic	Shirashi et al., 1992	
Bladder	Langkilde et al., 1989	
Lung	Kawai et al., 1991	
Ameloblastoma	Vigneswaran et al., 1993	
Melanoma	Kjonniksen et al., 1994	Nude
Sarcoma	Kjonniksen et al., 1994	Nude
Colorectal	Schumacher et al., 1994a	scid
Breast	Schumacher and Adam 1997	scid
Ovary	Schumacher et al., 1996a	scid

HPA as a prognostic marker in clinical studies

HPA and breast cancer

The ability of HPA to bind to cancer cells was first demonstrated in breast cancer by Leathem et al. (1983), being further clarified in two subsequent abstracts (Leathem et al., 1984, 1985; Leathem and Brooks, 1987). Leathem and his colleagues used formalin fixed, paraffin wax embedded biopsy material from 15 normal healthy controls and from 30 patients with breast cancer. They found that a panel of lectins including pokeweed mitogen (PWM), *Lotus tetragonolobus* agglutinin (LTA), wheatgerm agglutinin (WGA), peanut agglutinin (PNA), *Helix pomatia* agglutinin (HPA), *Bandeiraea simplicifolia* agglutinin I (BSA-I), soy bean agglutinin (SBA) and Concanavalin A (Con A) all bound to breast cancer cells in a variable, cytoplasmic, luminal surface or extracellular pattern. However, they did note that not all cancer cells bound the same lectin, suggesting the presence of sub-populations with different glycosylation pattern within individual tumours. With the exception of Con A, all lectins also bound to normal breast, specifically to the apical membrane of the lining epithelial cells or to the myoepithelial cells, and there were some differences in lectin binding between acinar and ductal epithelium. The main difference in the binding of lectins between normal and malignant cells was the transition from a surface to a mainly granular cytoplasmic binding pattern. The predominantly luminal pattern of lectin binding seen with most of the lectins used by Leathem and his colleagues suggested to them that there is a concentration of glycoconjugates in normal breast at this particular apical location, and was in accord with the demonstration of Franklin (1983), although in the latter study a different panel of lectins was used. Brooks and Leathem (1991) demonstrated a strong association between HPA staining in primary breast cancer and the presence of metastases in the local draining lymph nodes in a twenty four year retrospective study of 373 primary breast cancers (embedded in paraffin wax). In life tables calculated for lymph node status (metastatic versus non-metastatic) and HPA staining versus non-HPA staining patients were almost identical over 15 years follow up period. However, there was no association with tumour size, histological grade, S-phase fraction or age at diagnosis. In summary, it appears that whereas normal breast epithelium is apically HPA-positive, non-metastatic cancer is HPA-negative, and metastasising cancer is HPA-positive all over the tumour cell (Leathem et al., 1983).

Criticisms of the use of HPA as a marker of metastatic potential in breast cancer

There have been critics of the work of Leathem and Brooks (1987), and no universal agreement of the value of HPA as a prognostic indicator of the metastatic potential of human breast cancer exists (Galea et al., 1991; Taylor et al., 1991; Gusterson et al., 1993). In a study of HPA staining of 459 cases of primary breast cancers over a six year period, Galea et al. (1991) were

unable to reproduce the positive correlation of HPA as a predictor of outcome with a higher degree of reliability than the established prognostic indicators. They suggested that the differences may be accounted for because of the different lectin histochemical methods employed, and the source of the lectin itself. Indeed, such is the strength of their belief, despite this explanation, that they argued against the use of HPA in prognosis prediction, relying on their own negative findings and discounting the work of Leathem's group. Taylor et al. (1991) also argued against the use of HPA as a prognostic tool. In their study based on 363 primary breast cancers, these workers also were unable to establish a strong correlation between HPA staining and metastatic potential as revealed by analysis of lymph node involvement. Gusterson et al. (1993) studied 684 primary breast cancers from the International (Ludwig) Breast Cancer Study Group and found only a weak correlation between HPA staining and lymph node status. They contrasted the large advantage in disease free survival and overall survival for node-negative patients, with the situation for HPA-negative patients for whom there was no such advantage. However, Leathem and Brooks replied to their critics Taylor et al. (1991) and Galea et al. (1991), commenting that methodological differences certainly can account for the different results, emphasising that the indirect lectin histochemical technique is to be preferred to the direct technique as employed by their censors. As pointed out by Walker (1993), the level of sensitivity of HPA binding site detection seems to be critical in determining the prognostic significance. Furthermore, it has been demonstrated that methodology (including fixation and tissue processing) does indeed make a difference to the binding outcome (Brooks et al., 1996). In a twenty four year retrospective study on 373 cases of primary breast cancers using a direct and an indirect lectin histochemical technique, Brooks and her colleagues (1996) found that there was a considerable difference in the lectin binding pattern in individual cases using the two different methodologies. This finding is likely to be significant since Leathem's group used an indirect method whilst the others used a direct lectin histochemical technique. Indeed, in life table analyses to calculate survival advantage of HPA stainers versus non-stainers only the indirect method showed a highly significant correlation between HPA binding and survival. Hence, it may be difficult to compare the results of various studies when different methods of tissue preparation and different histochemical techniques have been employed.

Despite the cautionary note many other groups have found an association between HPA binding and prognosis in breast cancer. In a study of one hundred patients presenting during 1977 and 1979, followed up over a six year period, Fenlon et al. (1987) evaluated HPA binding to human breast carcinomas after trypsin pre-digestion of sections, and demonstrated a significant correlation of the staining to patient survival. Studies showed that the HPA binding related very well to lymph node status at the time of resection, and evaluation of the follow-up data indicated that patients with primary cancers with a greater proportion of cells staining with HPA had a shorter survival time. The work of Fukutomi et al. (1989) clearly indicated the value of HPA as a prognostic indicator: HPA staining was studied in 113 cases of primary breast cancers, 63 metastatic lymph nodes and 10 resected local recurrences demonstrating that HPA-positive tumours were associated with a poorer 5 year survival rate. Fukutomi et al. (1991) followed up their earlier work by correlating the prognostic value of HPA staining with gene amplifications in human breast carcinomas. In particular, the relationships between cell surface sugar chains of cancer cells and the amplifications of the proto-oncogenes c-myc, int-2, and c-erbB-2 were evaluated. It was found that the expression of the carbohydrates identified by HPA staining correlated with the expression of the proto-oncogene c-myc product. The prognostic significance of HPA staining and the oncoprotein c-erbB-2 were further evaluated in human breast carcinoma by Thomas et al. (1993) in 120 primary breast cancers. This group found that tumours which were both HPA- and c-erb B-2-positive were highly correlated to the presence of axillary lymph node metastases, a poorer overall and shorter disease-free interval compared to the other indicators considered alone. Investigation of the relationship between HPA staining and the presence of metastases in the axillary or internal mammary lymph nodes was strongly correlated, though HPA staining was unable to discriminate between the two lymph node groups. Flow cytometric studies of cell surface carbohydrates have also been made in metastatic breast cancer, including cell aspirates from primary adenocarcinoma and from draining lymph nodes with metastases (Alam et al., 1990). It was found that this technique allowed correlation between HPA staining and lymph node involvement in tumours of higher grade (II and III), and that the technique could be useful in analysis of fine needle aspirates of breast tissue. Noguchi et al. (1993) found that evaluation of HPA staining combined with DNA ploidy of breast carcinomas was a more powerful tool than use of either feature alone in the prediction of long term outcome in a study of 106 primary breast cancers. This was followed up by Leathem's group (Brooks et al., 1993). In this 24 year retrospective study paraffin wax sections of primary breast cancers from 366 patients were evaluated using HPA staining, histopathological assessment, and the S-phase fraction and ploidy calculated after flow cytometry. Data including the tumour size, nodal status and age were also recorded. It was found that the prognostic significance of altered glycosylation, as detected by HPA binding, is unlikely to be through an association with proliferative rate, degree of anaplasia, or cellular ploidy, but may rather be through a direct association with the presence of nodal metastases.

The ability of metastatic breast cancer cells to bind HPA seems to be a relatively constant feature since HPA

binding glycoconjugates are expressed in most brain metastases (Schumacher et al., 1992), and hence HPA may be regarded as a stable marker of metastatic potential. Such constancy of lectin binding between primary tumours and metastases was not a feature of other lectins applied to breast cancer tissues since Krogerus and Andersson (1990) did not observe a similar constancy of lectin binding site expression using a different panel of various lectins including *Bandeiraea simplicifolia* agglutinin II (BSA-II), Con A, HPA, PNA, *Ricinus communis* agglutinin (RCA-I or -II), *Ulex europeaus* agglutinin (UEA-I) and WGA. It was found that the binding pattern of the primary tumours was diverse, whereas in the respective metastases it was relatively homogenous. It was concluded that whereas there is heterogeneity within the primary tumours, the subclones of malignant cells which metastasised had a more restricted glycoconjugate expression. This view is in accord with the more general observations made by Kahn et al. (1988) who examined the lectin binding pattern of liver and lung metastasising variants of the murine Lewis lung carcinoma. They found that the lectin-phenotype of the metastasising cells depended on to which organ the cells were seeding. This implied that the selective metastasis formation was associated with differences in the carbohydrate composition of the metastatic tumour cells.

The carbohydrate profiles of primary breast carcinomas and their metastases has recently been investigated by Rye et al. (1993) in 36 primary breast cancers and associated axillary lymph nodes. Noting the well documented considerable heterogeneity of carbohydrate expression in malignant tissues, these workers were able to demonstrate almost complete homogeneity in carbohydrate expression between individual primary breast cancers and their lymph node metastases for all the lectins studied (WGA, PNA, LTA, HPA and UEA-I). Their findings led them to suggest that at this stage of metastasis more subtle changes in specific glycoconjugates are responsible for subsequent metastatic cell behaviour, rather than widespread changes in carbohydrate groups as usually detected by lectins. Indeed, Rye et al. (1993) have proposed that although the alteration in glycosylation associated with malignancy can be identified with lectins, the restricted specificity of lectins and the considerable array of potential binding sites on and within cells, means that any glycosylation differences in sub-populations of tumour cells are likely to be too subtle to detect using lectin histochemistry. For this reason Rye et al. (1993) regarded the use of lectins to be inappropriate and of limited prognostic value in breast cancer. However, this view is at variance with the earlier studies *(vide supra)* which showed a clear correlation between HPA staining and prognosis of breast cancer patients.

HPA binding and prognosis in other cancers

In advanced colorectal carcinoma, HPA staining has been associated with poor prognosis by Ikeda et al. (1994). In the latter work a total of 117 patients were studied, with 57 in one group who survived for at least five years, and 60 in a second group of patients who suffered a recurrence within two years or who died. The second group showed a greater intensity of HPA staining than the first, and there was a positive correlation in multivariate analysis between HPA staining, lymph node metastases, and degree of venous invasion. Schumacher et al. (1994a) also found significant correlation between positive HPA staining of the primary tumour cells and prognosis in colorectal carcinoma (n=130) which was correlated to the Duke's classification. The presence of HPA staining was found to be almost as bad as the prognosis for Duke's stage C, as predicted by conventional means. Of the Dukes' C stage patients 73% were also HPA-positive. The ability of HPA to distinguish between metastasising and non-metastasising cancers is quite unique. Using mistletoe lectin III (ML-III) which has the same nominal monosaccharide specificity as HPA, namely GalNAc, Dixon et al. (1994) could not find any prognostic value of this lectin in colorectal cancer. These findings were elaborated by analysing lectin binding sites on isolated membrane glycoproteins using Western blotting. ML-III bound to slightly different membrane glycoproteins as HPA. Hence, even subtle differences in the carbohydrate composition of glycoconjugates which can be differentiated by lectins are of functional significance with regard to the metastatic spread of tumours. These studies confirmed the earlier histochemical and Western blotting studies of Laferté et al. (1995) who reported that the combined use of monoclonal antibodies recognising blood group A and B oligosaccharides and HPA is able to detect glycoproteins specific for colon cancers in rats.

Poor prognosis has been associated with HPA staining in oesophageal carcinoma by Yoshida et al. (1993). In their study of 95 patients with primary oesophageal carcinoma (tissues collected over a 10 year period, 1980-1990) it was possible to relate positive HPA staining of the primary tumour to the progression of the tumour in terms of its infiltration into the depth of the oesophageal wall, and of the degree of venous invasion. Similar to other carcinomas the HPA-positive patients had a consequently poorer prognosis than those who were HPA-negative. These results were confirmed by a second publication from Yoshida et al. (1994) who studied the number of silver stained nucleolar organizer region (NOR) proteins and HPA staining in oesophageal carcinomas. They also found that HPA staining correlated significantly with increasing depth of the carcinoma invasion, venous invasion and tumour stage. Of the HPA-negative cases 40/42 also had low silver stained NORs, whilst 39/52 HPA-positive cases had NOR staining. The survival rate of patients with advanced carcinoma was significantly poorer for those with high NOR staining and HPA-positivity than in those with NOR staining and no HPA-reactivity. It was concluded that tumours expressing both prognostic

indicators represented a subgroup of carcinomas with a particularly poor prognosis. In contrast, a down-regulation of HPA staining in N-methyl-N'-nitro-N-nitrosoguanidine induced oesophageal carcinoma in shrews was demonstrated by Takahashi et al. (1994), *vide infra*.

In gastric carcinoma HPA has been found to be a useful prognostic indicator by Kakeji et al. (1991, 1994). In this work, HPA staining correlated well with other markers of tumour progression i.e. tumour size, penetration, lymphatic invasion, and metastasis. Indeed, those patients whose tumours were HPA-positive (96/163), and who had carcinoma cells on the serosal surface of their stomach, had a lower survival rate. In a study of 119 patients with gastric carcinomas, and with positive HPA staining (75/119) (for whom the 5 year survival rate was only 18%) there was also a higher rate of abnormal p53 staining (69% of the p53 positive cases were also HPA positive), and a higher concentration of proliferating cell nuclear antigen staining (38.5% of the PCNA positive cases were also HPA positive) (Maehara et al., 1995).

Prostatic carcinomas which had metastasised to bone showed a higher incidence of HPA binding than those which did not bind the lectin (Shirashi et al., 1992). These results indicated that those patients who were indigenous Japanese, rather than Japanese Americans or whites from Hawaii were more likely to bear tumours which were HPA-positive.

In invasive bladder carcinomas, out of a panel of nine lectins, only HPA stained the cancer cells, albeit a small percentage (8%) (Langkilde et al., 1989). Indeed, the overall finding was that the general decrease in lectin staining was accompanied by an increase in the progression of urothelial malignancy. This instance of down regulation of the glycotopes responsible for HPA binding may relate to the changes in the local environment, since the down regulation seems specific to the urinary bladder location.

HPA staining has been used to distinguish malignant pleural mesothelioma (n=29) from well differentiated pulmonary adenocarcinoma (n=38) (Kawai et al., 1991). Tissues from these two types of cancer were tested using HPA and also an antibody to ABH blood group related antigens. Reactive mesothelial lesions and malignant mesothelioma of the pleura were not stainable with the ABH antibody or HPA. However, in pulmonary adeno-carcinoma, the ABH antibody reacted with tissues expressing the compatible blood group, especially group O. HPA reacted with adenocarcinomas of blood group types A and AB (94% and 100% respectively), with less binding to type B (80%) and O (33%). It was suggested, therefore, that positive reactions with either the ABH antibody or HPA can be indicative of well differentiated pulmonary adenocarcinoma, and, thus, exclude mesothelioma.

HPA staining has been found to be useful in studying human germ cell tumours (Nikawa et al., 1993), though in these cases it appears to be a less powerful indicator of subsequent prognosis than in the tumours mentioned above. In patients with intra-cranial human germ cell tumours, in addition to HPA reactivity in neoplastic cells irrespective of blood group type, Nikawa et al. (1993) found that some vascular endothelial cells and erythrocytes of patients with blood group types A or AB were also labelled. HPA-positive neoplastic cells were seen in one yolk sac carcinoma in a patient with blood group type A, and in embryonal carcinomas and teratomas irrespective of blood group type. Although 10/18 germinomas were HPA-negative, 8/18 were HPA-positive. Whilst the former showed a very good response to radiotherapy, 4/8 HPA-positive cases showed poor radiosensitivity. In this instance the HPA reactivity was related to the degree of radiosensitivity of the tumours.

In 18 cases of ameloblastomas, ameloblastic carcinomas and basal cell carcinomas Vigneswaran et al., (1993) analysed their reaction to HPA. Of the 12 ameloblastomas 5 were HPA positive.

HPA is not, however, a universal marker of prognostic relevance since Schumacher et al. (1996) found that it bound, to varying extents, to all of a series of squamous cell carcinomas of the human aerodigestive tract in the head and neck region, and hence was of no use as a prognostic indicator (Fig. 1D).

HPA as a predictor of metastatic potential in animal models

It is possible to define the metastatic potential of human cancer cells in the clinical studies using HPA. This predictive power has been applied to model systems as well, which would have clinical relevance with respect to carbohydrate expression. In the work by Schumacher et al. (1994b) the human colon cancer cell line HT29 was injected sub-cutaneously into severe combined immunodeficient (scid) mice. Pulmonary metastases then rapidly developed in these mice spontaneously. Both in vitro and in scid mice the HT29 cells expressed HPA binding sites, though differences were noted (Fig. 1A,B). In investigations of the role of carbohydrates as revealed by lectin histochemistry in animal models of human breast or colon cancers, or in cancer cell lines grown *in vitro*, the carbohydrate expression associated with metastasis is modulated by local environmental differences: HT29 cells grown *in vitro* were more intensively HPA-positive and the staining was homogenous as compared to HT29 cells grown *in vivo* in scid mice (Schumacher et al., 1996c). Schumacher and Adam (1997) studied the lectin histochemical binding pattern of human breast and colon cancers that were associated with metastasis formation in scid mice. It was shown, in general, that those cancer cell lines which were HPA-positive metastasised, whereas those cell lines which were HPA-negative did not (Fig. 1C). This work also supported the use of the scid mouse model of metastasis formation, as reviewed by Mitchell and Schumacher (1997).

In a study of metastasis formation after intravenous

injection of human melanoma and sarcoma cell lines into nude mice (Kjonniksen et al., 1994), there was a relationship between the ability of the carcinoma cells to form metastases in the lungs, and HPA staining. Pretreatment of the cells with HPA prior to injection, however, did not reduce the ability of the cells to develop pulmonary metastases, indicating to Kjonniksten et al. (1994) that the pulmonary metastatic potential was not blocked by HPA, and hence the binding partner(s) of HPA was not directly involved in the metastatic process. A criticism of this methodology, however, is that it is not modelling metastasis formation, but simply a seeding via the vascular route.

A down-regulation of HPA staining in N-methyl-N'-nitro-N-nitrosoguanidine induced oesophageal carcinoma in shrews was demonstrated by Takahashi et al. (1994), as there was for other lectins in the panel tested. The reason for the down regulation is not clear, but the means of induction of the carcinoma may be important in relation to the effect on carbohydrate expression. Species differences may also be important.

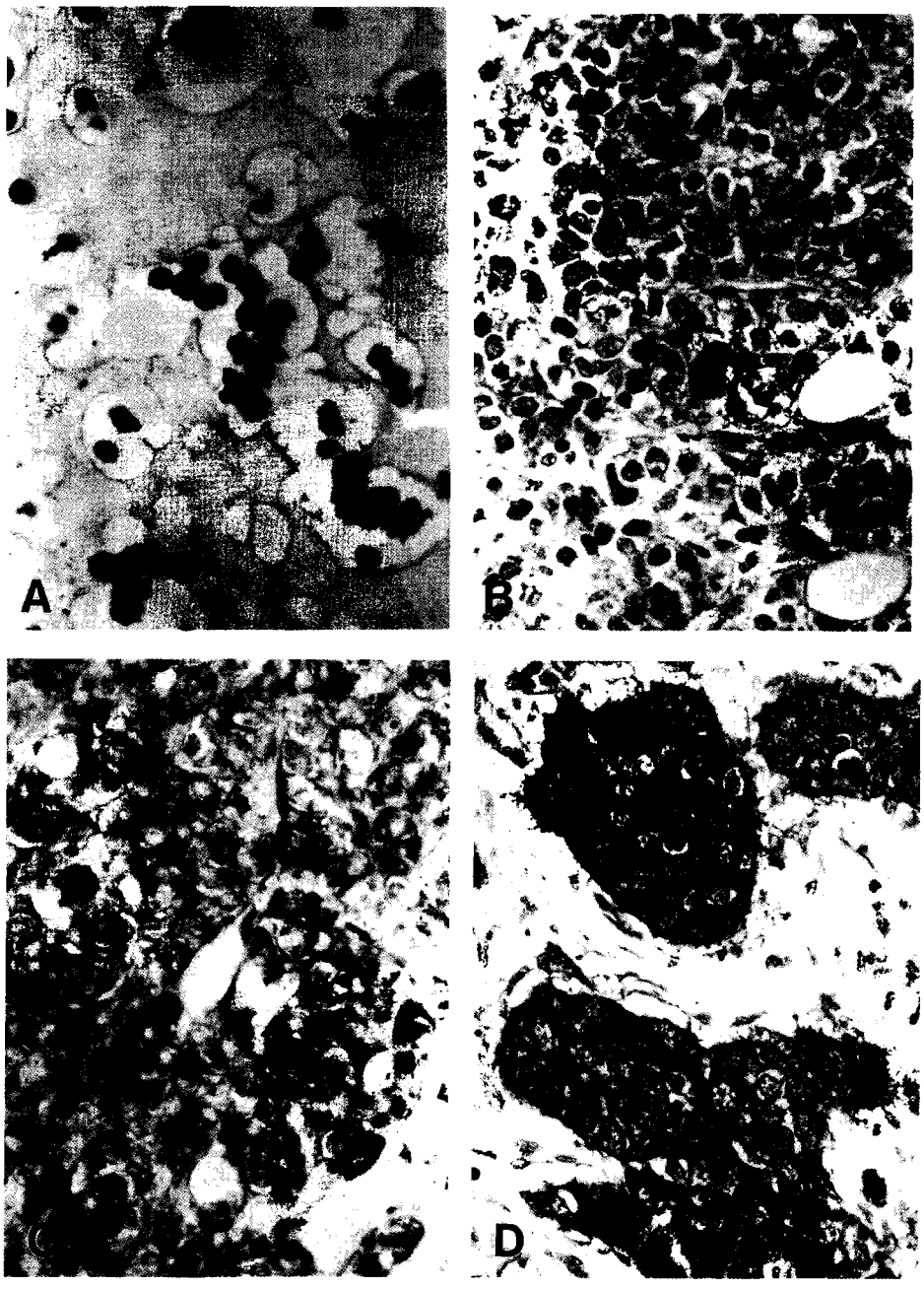

Fig. 1. A. The human colon cancer cell line HT29 grown *in vitro* and stained with HPA. Note the intense and homogeneous staining of the cells. **B.** HT29 tumour grown in scid mice. This tumour had metastasised to the lungs. Note the overall weaker and more heterogeneous staining pattern. This difference in staining intensity and pattern indicates that HPA binding sites are to a certain degree subject to cell regulatory mechanisms. **C.** HPA binding to the human breast cancer cells MCF-7 grown in a scid mouse. Again, a heterogeneity of the staining pattern is obvious. The tumour had spontaneously metastasised to the lungs. **D.** Human squamous cell carcinoma of the oropharynx stained with HPA. Note the intense membranous staining pattern. In this tumour type, however, HPA binding is not associated with metastasis. x 850

Identification of possible binding partners of HPA

HPA binding properties of several human breast cancer cell lines have been investigated (Schumacher et al., 1995), and it was found that lectin binding was very heterogenous between and within the different cell lines (and was influenced by the tissue fixation and processing). However, some of the cell lines showed HPA-binding sites both in sections and in isolated cell membrane glycoproteins using Western blots. The latter indicated that HPA can bind to several membrane glycoproteins. The binding pattern of HPA to most if not all membrane glycoproteins in breast cancer cells is of significance for the understanding of the glycosylation mechanisms in metastasising breast cancer. It should be borne in mind that HPA binds to the apical membrane of the normal breast epithelium only. The ability to bind HPA at all is lost in non-metastasising breast cancer, whilst metastasising breast cancer shows HPA binding sites on both apical and basolateral membranes. In addition, HPA binding sites are located in the cytoplasm of the latter tumour cells as well. To study this glycosylation pattern milk fat globule membrane glycoproteins were analysed. The milk fat globule membrane is a derivative of the apical membrane of the normal lactating breast epithelium. By analysing the HPA binding sites of these membrane proteins, only two to six membrane glycoproteins were detected, which bound HPA (Schumacher, 1990). Because almost all proteins of breast cancer cells bound HPA, it can be assumed that the apical-basolateral divide of HPA binding sites broke down in metastatic breast cancer cells indicating a switch in glycosylation.

Ultrastructural analysis of HPA binding sites of human breast cancer cell lines showed that after use of a pre-embedding technique HPA was bound to the typical submembranous granules and surface microvilli, the former representing sites of the milk fat globules (Mitchell et al., 1995). As to the molecular structure of the HPA binding sites, Brooks and Leathem (1995) have recently hypothesised that a putative binding partner of HPA was the Tn epitope, or the blood group A antigen, because in both cases there is an expression of glycoproteins terminating in α-GalNAc. This hypothesis was analysed using two monoclonal antibodies, one to Tn and another one to α-GalNAc and HPA as well. Only small proportions reacted for Tn or α-GalNAC, whereas 85% were HPA-positive. Indeed, the staining patterns indicated that the populations of cells demonstrated were different, and this was confirmed in Western blots. Thus, it was concluded that breast cancers may express complex carbohydrate profiles, but that distinct glycans sharing similar terminal GalNAc groups may be implicated in the development of the metastatic potential of breast cancer. Indeed, Ito et al. (1996) used a panel of lectins all binding to glycoconjugates with GalNAc residues in their backbone structures, and demonstrated that all the lectins bound to the same human breast carcinoma cells, and that all carried linear and branched poly-N-acetyl-lactosamine on N-glycans, suggesting that the synthesis of this complex carbohydrate is one of the most important and basic processes leading to the malignant transformation of cells, invasion, and metastasis of carcinoma cells. Another potential binding partner of HPA may be glycosaminoglycans. However, of all the glycosaminoglycans only hyaluronic acid has the potential of being a putative binding partner of HPA. Hyaluronic acid contains N-Acetyl-glucosamine residues, a carbohydrate for which HPA has a minor specificity. However, Schumacher et al. (1996b) have demonstrated that hyaluronic acid, at least, is not, in practice, the binding partner for HPA in breast and colon cancer. Hyaluronate digestion of tissue samples from human clinical and experimental tumours grown in scid mice showed that HPA bound to the tumour cells even after hyaluronidase treatment although HPA bound to hyaluronic acid in dot blots. Thus, it was suggested that the binding partner for HPA was more likely to be a GalNAc-containing glycoprotein.

The binding of HPA to tumour cells is subject to regulatory mechanisms. In general, HPA binding sites are more homogenous and intensive in breast and colon cancer cells grown in vitro, as compared to *in vivo* (*vide supra*). This variation of carbohydrate binding sites, however, is not unique to HPA but can also be found using the same cell lines with other lectins having different carbohydrate specificities (Schumacher et al. 1996c). These findings accord with the results of the work by Berezovskaya et al. (1995) at least for glioma cells maintained in vitro. The interaction of the cancer cell with its environment is mediated by cell membrane glycoprotein receptors. In the glioma cell lines it was shown that the increase in the degree of sialylation or branching of fucose-containing residues could influence cell adhesion properties, thus creating metastatic potential. Indeed, the degree of sialylation of the subterminal galactose or GalNAc residues shows a good correlation with metastatic potential (Altevogt et al., 1983; Yogeeswaran and Salk, 1991) amongst various murine metastatic tumour cell lines.

HPA staining correlated to other lectin markers of metastatic potential in clinical studies

Another lectin which has been associated with metastatic potential in carcinomas, and hence a poor prognosis, is *Phaseolus vulgaris* leucoagglutinin (PHA-L). It binds to a different group of carbohydrate residues, namely ß1-6 branched oligosaccharides. The malignant transformation has been associated with the acquisition of larger asparagine (N)-linked oligosaccharides (Warren et al., 1978), and GlcNAcß(1-6)Manα(1-6)Manß1-6 branching asparagine-linked oligosaccharides in human breast and colon carcinomas (Fernandes et al., 1991). Fernandes et al. (1991) have found a correlation between prognosis and PHA-L binding to primary human breast and colon cancers. Indeed, Dennis and Laferté (1989) had earlier demonstrated that PHA-L bound most

strongly to malignant rather than benign breast tissues. Recently, Mitchell et al. (1998) compared the binding pattern of HPA and PHA-L in parallel sections of a series of experimental colon and breast cancers grown in SCID mice, as well as in clinical material from patients with breast cancer. The histochemical reactions obtained with the two lectins resulted in very different staining patterns. Indeed, it was shown that HPA was more often associated with metastases than PHA-L. The results indicated that a common complex type glycoprotein terminating with a GalNAc residue is, therefore, unlikely to be the binding partner for both lectins.

Conclusions

It is clear, despite some contrary views, that HPA has some relation to the survival of patients in a variety of cancers mostly of the adenocarcinoma type. The binding partner of HPA is unknown, though it appears to contain α-GalNAc or GlucNAc residues in a glycoprotein. The expression of HPA appears to correlate with a number of parameters including metastasis formation, and the expression of certain oncogene products. In multivariate analysis, it is better correlated with patient prognosis than with tumour size or differentiation. The expression of HPA depends on the techniques used for its demonstration, and on whether cancer cells are examined *in situ* or after *in vitro* culture. Future studies of patient prognosis should be aimed at elucidating the role of the carbohydrate binding partner of HPA, and its role in the metastatic process. Recent progress in molecular biology may direct the way in which an analysis of HPA binding sites may be fruitful. In comparing the gene expression of at least 45000 different genes from normal colonic mucosa and colon carcinoma Zhang et al. (1997) found remarkably few differences between the two samples. One gene, whose expression was downregulated, however, was a sialyltransferase. If it can be shown that HPA detects a subterminal carbohydrate residue in metastasising colon cancer cells, which is normally masked by sialic acid, a remarkable step forward in the understanding of the metastatic cascade will have been made. Current work in our laboratory is now focusing on this promising path to elucidate the role of this sialyltransferase activity to mask HPA binding sites in non-metastasising cancers.

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