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Immunohistopathological Characterization of Spontaneous Metastases in a Human Lung Mucoepidermoid Adenocarcinoma (HLMC) Xenograft

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The most common clinical form of lung cancer is a disseminated disease with distant metastases; several years of cancer progression precede presentation, and this ultimately limits the efficacy of curative therapy. In this immunohistochemical study, we examined a mucinous adenocarcinoma cell line, maintained by xenogeneic transplantation, and a spontaneous metastatic variant which produces distant tumors (in liver, spleen and kidney). The aim was to investigate possible parameters which characterize the metastatic process. Histopathological comparison between the two subcutaneous transplanted tumor lines showed that both lines presented a similar cellular morphology, a different pattern of cellular growth and an increased vascularization in the metastatic line with respect to its parent. All the tumor sections expressed differential immune reactivity with monoclonal antibodies against Lewis y (MAb C14), sialyl-Lewis x (MAb SNH3) and Lewis x (MAb FH2) determinants. Neither expressed MUC 1 mucins detectable with monoclonal antibodies reactive with the mucin protein core (MAbs C595 and SM3) nor was carcinoembryonic antigen (MAb C365) expressed. Neoplastic cells were reactive with an anti-pan cytokeratin monoclonal antibody confirming their epithelial histogenesis. Our findings have been evaluated with respect to defining metastatic phenotypes in lung cancer by examination of distinct histopathological and immunological parameters. (Pathology Oncology Research Vol 4, No 4, 259–266, 1998)

Key words: immunohistopathology, metastases, lung adenocarcinoma xenograft

Introduction

Lung cancer is one of the most common and lethal forms of malignant disease. Metastatic spread, even before diagnosis of the primary tumor, as well as inefflcacy of surgical and chemotherapeutic strategies account for poor prognosis The 5-year survival rate is less than 15%[31] and about 80% of the patients die of tumor recurrence after surgical resection with distant metastases in brain, lung, adrenal, liver and bone.

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Tumorigenic lines in athymic nude mice have been developed to study human cancer cell biology and for testing new antitumor strategies. The predominant prototype in this approach is the subcutaneous xenograft[14,45] although other propagation models have been produced.[1,4,29] Several reports[5,6] have indicated that the presence of a primary tumor inhibits the growth rate of its metastasis or of a second tumor implant. Metastatic dissemination depends largely on the propagation methods employed, but the spontaneous development of metastasis from a xenotransplanted tumor is not a frequent event.

Several mechanisms and factors have been associated with the invasive and metastatic events: expression of different oncogenes,[17,54] detection of several polypeptides or amines,[20,34] production of diverse enzymes,[46,55] differential expression of some adhesion molecules,[8,36] as well as the

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ability of tumor cells to interact through their adhesion receptors with extracellular matrix or with other cell type surfaces.[19] These molecular events reflect several histologic changes such as loss of cellular polarity and diminished intercellular contacts, decrease of apoptotic events, increase in tumor vascularization, presence of tumor cells gaining access to vessels and infiltration of normal tissues.

Metastatic spread is also accompanied by changes in membrane antigen expression at the different sites of tumor localization.[11] This altered expression of antigens has also been observed in lung cancer[12,53] and moreover, it has been noted that blood-group antigen expression (e.g., H/Lewis y/Lewis x) may relate to the survival of patients with non-small-cell lung cancer.[24,32]

The aims of the present investigation have been as follows: (1) to establish a comparative histopathological description of a subcutaneous xenografted line of a human lung mucoepidermoid carcinoma (HLMC) and its spontaneous metastatic variant, and (2) to investigate the expression of different tumor antigens in these malignant tissues in order to identify the putative surface receptors or markers which may be involved in neoplastic cellular processes in the development of metastases.

Materials and methods

Human lung tumor xenograft line

Establishment of the human lung mucoepidermoid carcinoma (HLMC) xenograft line 8430/3-ICD-O has been described.[33] Viable cells (1 to $1.5x10^6$) were implanted subcutaneously in the subaxial area of 6 to 8 week old athymic nude mice. The HLMC tumor line has been maintained for the past 15 years by serial subcutaneous transplantation using 1500–2000 mm^3 tumor fragments. Recipient animals were kept in a pathogen free environment, all experimental procedures were performed in a class B laminar flow hood. Monitoring of arllmals for tumor development was performed daily and the presence of metastasis was examined at each transplant generation.

Measurement of tumors

Xenografted subcutaneous tumors from the original xenograft line were measured twice weekly and the volume was expressed as relative volume respect to the initial volume.[7] Metastatic spontaneous xenografted line tumors were measured daily and sizes calculated according to Helson et al.[21]

Immunohistochemistry

All specimens were fixed in phosphate buffered formalin, embedded in paraffin and cut into 5 pm serial sections. Deparafftnized sections were cleared with xylene and rehydrated in 100, 90, 70, 50 and 20% alcohol solutions, washed twice with phosphate buffered saline (PBS, pH 7.2) and treated with 0.3% hydrogen peroxide in absolute methanol for 30 min to block endogenous peroxidase activity. After washing with PBS, sections were incubated for 30 min with 10% human serum in PBS containing 1% bovine serum albumin (BSA) in order to block non-specific immunoglobulin binding. Sections were then incubated for 1 hr with mouse monoclonal antibodies (at 10 μg/ml or as hybridoma supernatants); after washing with PBS, sections were incubated for 1 hr with peroxidase conjugated rabbit anti-mouse immunoglobulins (1:400, in PBS containing 1% BSA). They were washed with PBS and finally reacted for 10 min with 0.05% diaminobenzidine substrate and 0.01% hydrogen peroxidase in PBS. After rinsing in water, slides were stained with hematoxylin and mounted. Negative controls were either incubated with PBS or non-immune mouse serum instead of the monoclonal antibodies. All steps were performed at room temperature.

The whole area of each tissue sample was observed by sequentially examining lower power (x10) optical fields, the staining of cytoplasm, plasma and nuclear membranes and secretory products were separately evaluated. Cells were considered to be positive when at least one of these components was positive; heterogeneity was graded according to positivity, intensity and distribution.

Staining intensity was graded as negative (–), moderate (+) or strong (++). The number of low power (x10) optical fields in a specimen that were positively stained (+ or ++) was expressed as a percentage of the total number of optical fields containing tissue. A specimen was considered focally or diffusely positive if immunoreactive cells were found in 5% and 20% of low-power fields, respectively. Hematoxylin, eosin and periodic acid-Schiff stained sections were evaluated routinely. All tissue samples were coded before immunohistochemical analysis.

Image processing and analysis

A quantitative analysis of the areas of immune reactivity with different antibodies, related to total tissue area, was performed, For this analysis, histological images were captured from an Olympus BX50 system microscope (Tokyo, Japan) with an objective magnification of x40 through a video camera (Sony DXC-1 S 1 A CCD color video camera, Tokyo, Japan) and digitized with a 512x480-pixel resolution and a 24 bit true-colour monitor, TIFF format (PENTIUM, Samsung color monitor), SNAPplus desktop video adapter (Cardinal Technologies, Inc., Lancaster, PA, USA), software: Image-Pro Plus for Windows v 3.5 (Media Cybernetics, Silver Spring, MD, USA). To separate the immunostain (brown stain) from the hematoxylin stain (blue stain) the Color Segmentation

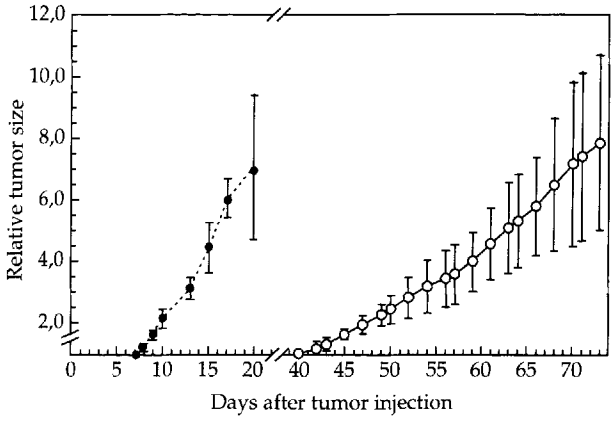

Figure 1. *Growth rate of human lung mucoepidermoid carcinoma xenografted in nude mice. (O) original subcutaneous line, (●) metastatic subcutaneous line. Values represent the mean of 10 animals per group +/- S.E.*

operation was applied following Portiansky and Gimeno.[39] Fields of vision showing inflammation, necrosis or nuclear pyknosis were excluded.

Monoclonal antibodies

The following monoclonal antibodies (MAbs) were employed: MAb C14, an IgM anti-Lewis y hapten, against the difucosylated Type-2 blood group chains,[9] MAb SNH3, an IgM anti sialyl-Lewis x, and MAb FH2, an anti-Lewis x. Two anti-MLJC1 mucin MAbs were employed: MAb C595 (IgG3) defines the tetrameric epitope Arg-Pro-Ala-Pro in the MUC1 protein core[42] and MAb SM3 (IgGl) reacts with the MUC1 protein core epitope Pro-Asp-Thr-Arg-Pro, which is selectively exposed in MUC1 expressed by breast, ovarian and other carcinomas,[49] MAb C365 (IgGI) is specifically reactive with carcinoembryonic antigen (CEA),[40] the anti-Pan Cytokeratin MAb (IgG) (Code C-2562, Sigma Chemical Co., USA) was also employed.

Results

Tumor growth rates

The average growth rates of different subcutaneous transplant generations are depicted in *Figure 1*. The latency period was about 30 to 45 days, with a tumor take rate of about 80%. The HLMC xenograft line has remained consistent over 15 years as a moderately differentiated lung adenocarcinoma with production of mucinous material. Tumors were localized at the inoculation site without invading surrounding areas, forming a large spheroid mass, usually containing a necrotic core with ulceration of the skin in advanced tumors.

When the original tumor acquired metastatic potential, rapid tumor development was observed at the site of the subcutaneous inoculation, the latency period was reduced to 7 days with a tumor take rate of 100 %. *(Figure 1)*. With the metastatic phenotype, a markedly different pattern of growth was obsemed: an expansive tumor developed in the subcutaneous tissue with infiltration in the skin. There was rapid involvement of the surrounding areas to the opposite limb and neck. Multiple hepatic, splenic and renal metastases were found in the first and successive transplant generations of subcutaneous implants .

In section, both subcutaneous xenografted lines showed a pale and soft gray-white colour with apparent mucinous secretion and lacking capsule. The metastatic spontaneous line showed a considerable increase in vascularization that was evident macroscopically.

Histopathological description of the xenotransplanted tumors

The original xenografted line *(Figure 2)* consisted of a moderately differentiated mucoepidermoid adenocarcinoma. The cells grew in nests composed of two different cellular types, first, basal and large cells with clear cytoplasm and delicate nucleus; – sometimes thesecells lined the nests with few intercellular bridges. A second type of small, round and more chromatic cells was also present; – these cells had small nuclei with dark chromatin, and necrosis and apoptotic elements were frequently observed towards ahe center of cell nests. Occasionally the epithelial elements were identified in acini together with amorphic material that reacted strongly with periodic acid-Schiff (PAS) stain. This stain also highlighted apparent

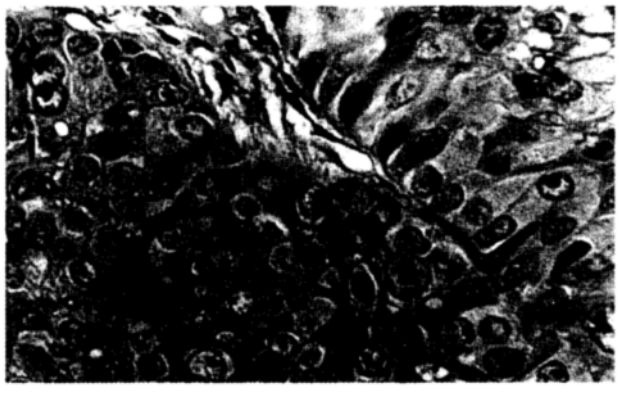

Figure 2. *The original xenografted line is a moderately differentiated, mucus-secreting adenocarcinoma. To the right, large cells with hypochromatic nuclei and clear cytoplasm are shown. These cells were frequently disposed at the periphery of tumor and in contact with connective tissue. To the left, small and dark cells with reduced nuclear mass and cytoplasm are in admixture with cells of the larger type – in both cell types nuclear pleomorphysm and anisocytosis are found (x400).*

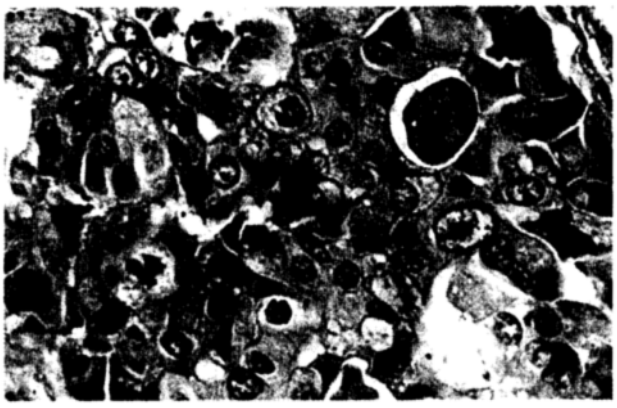

Figure 3. Metastatic subcutaneous tumor line. Small and large, as well as condensed and clear nuclei with abnormal nucleoli are shown indicating tumor atypia. Cytoplasmic vacuolae and cellular heterogeneity are present (x400).

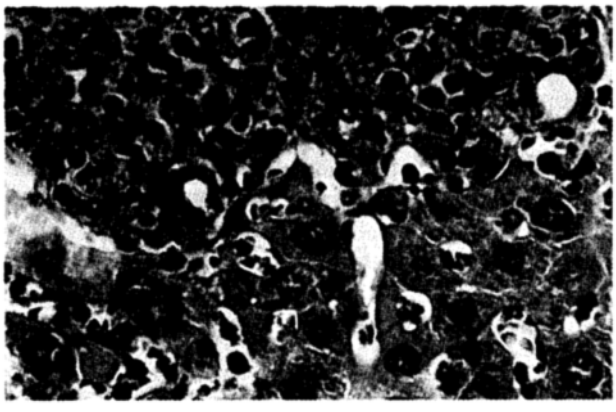

Figure 4. Section of the invasive front of an hepatic nodule from the subcutaneou metastatic line. Two areas of interest are illustrated: towards the top, tumor cell invasion is evident with cells showing the indistinct polarity and infiltrative growth pattern. Towards the left at the bottom, tumor cells in a vascular space can be identified (x400).

mucus secreting cells and both large and small mucus-filled vacuoles were observed.

The subcutaneous tumor from the metastatic xenografted line *(Figure 3)* showed similar cellular morphology. However, some differences were noted. The tumor grew as an expansive and polymorphic mass with a more evident host vasculature supply. This vasculature consisted of two or three medium arteries subdivided in many small vessels around the tumor mass. The core tumor vessels presented a microscopically chaotic architecture, they were tortuous with an abnormal lumen size and shape, and they were often covered by large endothelial cells. Furthermore, invasion of perivascular spaces as well as blood vessels was readily seen. Other histological differences could also be detected in the metastatic line: the nests and cords were not present while the dominant histological feature was the mixture of large and small cells growing without order and with frequent necrotic cells in the center of the tumor mass. Cells with large cytoplasm and nuclei at the periphery were frequently observed – apoptotic elements disseminated throughout the tumor mass were occasionally noted.

In both the original and metastatic subcutaneous xenograft lines, stromal components were non significant, abundant mitotic figures and cytologic atypia as well as nuclear pleomorphism were observed.

Metastatic neoplastic tumors were detected in liver *(Figure 4)*, kidney and spleen, they consisted in multiple foci of both large and small neoplastic cells without capsule or fibrosis infiltrating the periphery with the presence of many capillary vessels invaded by malignant cells, predominantly at the margins of the nodules. Occasionally, tumor cells formed a uniform and continuous line of cuboidal or low columnar adjacent cells. In the case of hepatic nodules, malignant cells grew around a central vein, from where they spread into normal hepatic tissue. The appearance of nuclei were very similar despite their metastatic character, they were large and vacuolated and also contained one or more large nucleoli. Small and hyperchromatic nuclei were present mostly in small cells.

Immunohistopathological studies

All HLMC tissue sections from the subcutaneous tumors from the original and metastatic xenografted cell lines as well as the metastatic nodules from liver, spleen and kidney were analyzed by for reactivity with a panel of monoclonal antibodies (*Table 1*), they were reactive with MAbs against Lewis y (C14), sialyl-Lewis x (SNH-3) and Lewis x (FHZ) with the exception that splenic metastases did not stain with FH2 MAb.

A common feature of all the tumor specimens was heterogeneity in staining with all MAbs tested: complete staining of the total specimen was never observed.

Tumors implanted subcutaneously from the original xenografted line reacted with the C14 MAb *(Figure 5)* and

***Table 1.* Immune reactivity of anti-tumor monoclonal antibodies with tissues from HLMC xenografts and metastatic varians**

Tissue	*C595*	*C365*	*C14*	*SM3*	*SNH3*	*FH2*
Subcutaneous parental xenografted line	<5%	<5%	37.8%	<5%	48%	25.3%
Subcutaneous metastatic xenografted line	<5%	<5%	7%	<5%	19%	7.0%
Renal metastasis	<5%	<5%	30%	<5%	66.3%	6.8%
Hepatic metastasis	<5%	<5%	16%	<5%	25%	13.5%
Splenic metastasis	<5%	<5%	9.4%	<5%	7.9%	7.3%

Staining intensity: (–): negative, (+): moderate, (++): strong.

Figure 5. Staining of the original subcutaneous line with anti Le y (C14) MAb. Heterogeneous and granular positive staining throughout the cytoplasm as well as plasma and nuclear membrane staining are shown (x400).

Figure 6. Staining of the metastatic subcutaneous tumor line anti-sialyl Le x (SNH3 MAb). Cells showing both strong staining and little or no staining are shown. Positive staining is predominantly localized to the plasma membrane and surrounding nuclear mass (x400).

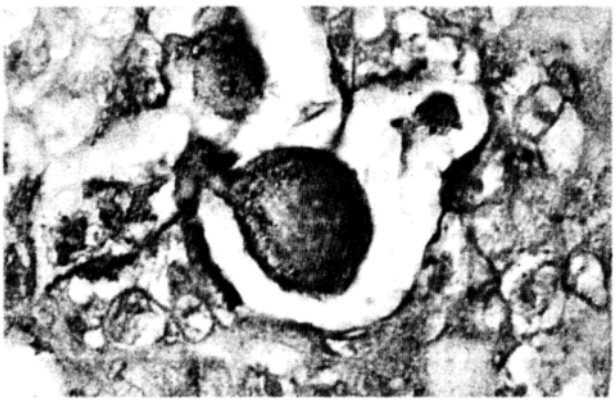

Figure 7. Hepatic tumor nodules stained with anti-Le y (C14) MAb. Strong positive reactivity is observed at cell membranes with some cells showing a linear pattern. Immune reactivity in the cytoplasm in a granular disposition is found mainly around nuclei (x400).

anti-sialyl-Lewis x (SNH3) with predominantly vesicular cytoplasmic staining of some cells in the nests; their nuclear membranes were also reactive and some apical membranes in several tumor acini reacted strongly with a granular pattern. The anti-Lewis x (FH2) MAb displayed a similar pattern of reaction but with reduced intensity. Subcutaneous tumors of the metastatic xenograft showed immune reactivity predominantly at the cellular membrane with the anti-sialyl-Lewis x MAb *(Figure 6)*. A reduced level of staining with anti-Lewis x and the anti-Lewis y MAbs was observed.

Neoplastic cells were reactive with the anti-pan cytokeratin MAb confirming their epithelial histogenesis.

Metastatic cells in liver nodules stained with the anti-Lewis y MAb – reactivity was very strong in cellular membranes with moderate cytoplasmic staining in some cells *(Figure 7)*. These hepatic metastases also showed a discrete cytoplasmic reaction with the anti-sialyl-Lewis x MAb (SNH3); the anti-Lewis x Mab (FH2) displayed a lower level of staining. Discrete areas of the splenic nodules showed a moderate reaction with the SNH3 MAb, the reaction was mainly at the cytoplasmic level although some cells showed membrane staining.

Only the cytoplasm showed a weak reaction with the anti-Lewis y MAb (C14). Conversely, large cells from the renal metastatic nodules were strongly reactive, particularly with SNH3, which showed positive membrane staining. A homogeneous, weakly positive cytoplasmic reaction was observed in some areas. The pattern of staining with C14 varied within the areas examined, in the core of the tumor nodule the reaction was strong in peripheral cytoplasm regions and in cellular membranes while in other areas weak reactivity was restricted to the cytoplasm. In other areas, strong staining of cellular membranes and weak cytoplasmic staining was found. It was also possible to detect immunoperoxidase reaction with the anti-Lewis x MAb in some restricted areas of these renal implants, exclusively located in the cytoplasm.

Quantitative immunohistochemistry

Standard methods were confirmed by the quantitative immunohistochemical analysis. The highest percentages of stained area in relation to the total tumor specimen were obtained with the anti-sialyl-Lewis x MAb (SNH3) showing 66.3% for the renal metastatic nodules, 48.0% for the parental line, 25.0% for the hepatic nodules and 19.0% for the subcutaneous metastatic line; the splenic tumor nodules showed 7.9% positive staining. Percentages obtained with the anti-Lewis y MAb (C14) showed the highest levels in the parental line (37.8%) and in the renal metastatic nodules (30.0%); the hepatic implants revealed lower reactivity (16.3%), the splenic nodules as well as the metastatic subcutaneous line showed the lowest levels of

staining at 9.4% and 7.0%, respectively with this MAb. Incubation with FH2 MAb revealed a lower levels of positivity with 25.3% in the parental line, as the maximal level; the hepatic metastases showed 13.5% of reactivity area while the other localizations were lower than 10%. Finally, anti-MUC1 as well as anti-CEA MAbs showed the lowest levels of staining which did not exceed 5% in any case.

Discussion

We have performed a comprehensive histological and immunohistochemical analysis in two variants of a xenografted HLMC tumor, the original and the metastatic lines as well as an examination of the metastatic nodules at different locations.

A number of subtle changes must occur during the acquisition of a potential for invasion and metastasis formation, producing a geno/phenotype which may be similar to its non-metastatic counterpart but sufficiently different to colonize distant tissues. One crucial step in tumor metastasis formation is the establishment of a vascular supply from the host;[15] formation of new vessels (angiogenesis) is also an essential process for tumor growth.[51] Recently, correlations between tumor vascularization and metastatic risk have been demonstrated,[26,37] since blood vessels provide the most important escape route for disseminating tumor cells. In the two xenografted cell lines considered here, a striking difference in vascularization was observed, the parental line appeared with a poor development of vasculature while in the metastatic line, increased host and tumor vessels were present.

Different histological characteristics were found when the subcutaneous tumor lines (parental and metastatic) and the metastatic nodules were compared. The original subcutaneous tumor showed nests of columnar tumor cells with more glandular aspects. Conversely, the metastatic variant presented as a more compact, undifferentiated and homogeneous mass without glandular features.

The expression of a number of tumor antigens and cell surface determinants has also been related to metastatic behaviour since they may reflect or contribute to functions such as adhesiveness, motility and invasiveness. In the present immunological evaluation we have investigated the expression of antigens and determinants in metastatic deposits at various tissue locations in tumors with defined patterns of growth. Notably, while the parental HLMC line expressed mainly Lewis y and sialyl-Lewis x antigens, and to a lesser extent, Lewis x determinants, differences in this antigenic expression were observed in the subcutaneous xenografts of the metastatic line as well as in the metastatic hepatic, renal and splenic tumors.

In the last decade many carbohydrate determinants shared by glycolipids and glycoproteins have been described,[16,18] these determinants were found to be accumulated in various tumors and could be defined by the monoclonal antibodies. Moreover, some of these carbohydrate antigens not only are present in cancer cells but can also be released into the circulation since Lewis x, Lewis y, poly-Lewis x and sialyl-Lewis x determinants have been detected in serum from patients with tumor at diverse tissue locations including lung cancer.[23,32]

The Lewis y determinant, a difucosylated type 2 blood group antigen, is a positional isomer of the Lewis a fucosylated derivative of the Lewis x antigen.[18] Kim and his colleagues[25] concluded that in human colon, the Lewis y hapten is an oncodevelopmental tumor-associated antigen and extended Lewis y antigens are highly specific markers for malignancy and premalignancy in this malignant disease. The C14 monoclonal antibody,[9] employed in the present investigation, recognizes the Lewis y hapten (difucosylhexose)[41] and staining of colon cancer tissues correlated with the host's ABO status.[10] Recently, studies have addressed the probable importance of Lewis y expression in relation to cellular motility.[32]. Moreover, expression of this hapten has been related to differentiation and apoptosis [30] and it was suggested was that cells expressing Lewis y proceed to apoptosis by induction of differentiation.[52]

In the present study, the Lewis y hapten was extensively expressed in the original subcutaneous line while a lower reactivity was detected in the other malignant tissues analyzed. It is also important to emphasize that in the original HLMC line considered here, apoptotic features were frequently observed which is consistent with a relationship between these observations and Lewis y expression.

In recent years, it has been possible to establish that some of these carbohydrates are expressed by leukocytes and mediate their adhesion to endothelial cells.[3] The carbohydrates sialyl-Lewis x and sialyl-Lewis a are known to be ligands for the cell adhesion molecule ELAM-1 (E-selectin, endothelial cell leucocyte adhesion molecule-I)[38,50] and they are of particular importance in the adhesion of human epithelial cancer cells to vascular endothelium.[47] The Lewis x antigen has been related to L-selectin adhesion that can also be present in the endothelial vessels and may function as the sialyl-Lewis x.[44] In the present study, the sialyl-Lewis x was the principal antigen expressed in subcutaneous tumors of the metastatic line and was also reactive in the metastatic deposits suggesting a close relation with the tumor dissemination.

Some of the carbohydrate ligands considered above have been described linked to the protein core of epithelial mucins[48] and members of the CEA family.[2] Aberrant expression of mucin and mucin-related antigens has been considered to be a poor survival factor in adenocarcinomas and carcinomas arising from various organs, such as breast and colorectal cancer.[22,28,48] Experimentally, mucins have been shown to promote tumor cell invasion and metasta-

sis.[13] However, in the present study, there was no associatiori between MUC1 or CEA expression and tumor growth and metastatic potential.

The subcutaneous tumor from the metastatic xenografts generally displayed a lower immune reactivity compared with the parental line and some metastatic deposits. This is in accord with previous observations showing that metastatic lesions may contain more carbohydrate antigen-positive cells than the original tumor[27] suggesting an association or role for these antigens in metastasis. Moreover, expression of H/Lewis y/Lewis b antigens have been correlated with survival in patients with carcinoma of the lung.[32] Similar results have been obtained in human colonic cancer since the presence of sialyl-Lewis x, sialyl-Lewis a and sialosyl-Tn in the primary tumor strongly correlated with patient survival.[35]

The pattern of carbohydrate antigenic expression may also correlate with the site of metastasis. We determined that Lewis y antigen was expressed at higher levels in hepatic deposits while sialyl Lewis x was expressed at higher levels in renal and splenic tumors. Since it is probable that hematogenous metastasis occur in HLMC dissemination; potential explanations could be related to the heterogeneous antigenicity in tumor cells according to the site of metastasis as well as the difference in the surface antigen density. It can be also postulated that alterations in the synthesis of these two antigens may also be related to the different sites of metastasis.[43]

In conclusion, the present study has shown that both xenografted tumor lines (original and metastatic) presented a similar cellular morphology, a different pattern of cellular growth and an increased vascularization in the metastatic line compared with the original. A quantitative differential expression of carbohydrate antigens was detected using a panel of monoclonal antibodies. These observations reflect the diverse phenotypes which permit the distinct organ colonization of malignant lung cancer.

Acknowledgements

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