Antigen expression of metastasizing and non-metastasizing human melanoma cells xenografted into nude mice

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In order to study differences in antigen expression related to the different stages of the process of metastasis of human melanoma cell lines, we determined the expression pattern of a series of well-characterized genes in a set of human melanoma cell lines with different metastatic behavior in nude mice. This set included non-metastatic (IF6, 530), sporadically metastatic (M14, Mel 57), and frequently metastatic (BLM, MV3) cell lines after subcutaneous inoculation. To study the phenotype of these cell lines both the cultured cells and representative samples of local tumors at the inoculation site and their metastases in the lungs were immunostained with a panel of monoclonal antibodies directed against melanocytic differentiation or progression antigens. Although most cell lines (IF6, 530, M14 and Mel 57) showed HLA-DR expression *in vitro*, these antigens were lacking in all xenografted lesions studied with exception of the 530 cell line. 530 Xenografts, however, showed a dramatic down-regulation of HLA-DR compared with the cell line *in vitro*. The same phenomenon was seen with respect to ICAM-1 expression. The expression of all other antigens studied in xenografts, both in subcutaneous tumors and in lung lesions, was in general comparable to that in the melanoma cell lines *in vitro*, with exception of the 530 cell line. In all melanoma cell lines except 530 the degree of intra- and interlesional heterogeneity regarding the expression of all antigens studied was limited. Remarkably, comparison of the immunophenotype of the frequently metastasizing (BLM, MV3) and the sporadically (M14, Mel 57) or non-metastasizing (IF6, 530) cell lines showed that the two frequently metastasizing cell lines had marked expression of the progression antigens VLA-2 and epidermal growth factor receptor, and lack of expression of the differentiation antigen NKI-beteb. These findings warrant further studies on the role of these antigens in the process of metastasis of human melanoma cells in nude mice.

Introduction

A number of monoclonal antibodies (MAbs) have been developed that recognize antigens expressed by human melanocytic cells. These melanoma-associated antigens (MAAs) include differentiation antigens, progression antigens and other antigens, such as functional antigens. Differentiation antigens are

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expressed both in benign and malignant melanocytic lesions while progression antigens preferentially are expressed in one or few stages in the process of tumor progression [3, 12, 30, 31].

The various phenotypic profiles of the different stages of tumor progression in human melanocytic lesions have been obtained by compiling the data of large numbers of different lesions derived from different patients. In order to be able to study subsequent stages of tumor progression in one individual we have chosen to use the nude mouse model employing human melanoma cells. A second important reason to use the nude mouse in research on tumor progression is that genetic manipulation (e.g. transfection experiments) can be performed to study the role of candidate genes in the pathogenesis of metastasis in an *in vivo* system. For these purposes we decided to use a panel of human melanoma cell lines with different biological behavior in nude mice. This approach seemed feasible as some human melanoma cell lines were shown to be highly metastatic after subcutaneous (s.c.) or intravenous (i.v.) inoculation [10, 17, 24].

Based on the foregoing we have studied the immunophenotype of metastasizing and non-metastasizing human melanoma cells xenografted into nude mice using a set of MAbs directed against MAAs. In particular we looked for differences in antigen expression (1) between the cell lines *in vitro* and *in vivo* (cultured cells vs s.c. tumors in nude mice), (2) between s.c. tumors and lung metastases in nude mice, and (3) between the different cell lines in relation to their metastatic behavior in nude mice.

Materials and methods

Nude mice

BALB/c athymic nude mice (nu/nu) were purchased from the Laboratory Breeding and Research Center, Gl. Bomholtgaard, Ry, Denmark or bred in the nude mouse facility of the Central Animal Laboratories, University of Nijmegen. Animals were kept in separate rooms in cages covered with air filters under specific pathogen-free conditions. The mice were used when 4–6 weeks old. Within a single experiment the mice were sex and age matched.

Human melanoma cell lines

BLM is a subline of BRO [24] (gift from Dr B. Giovanella, Stehlin Foundation, Houston, TX), isolated from a lung metastasis produced after s.c. inoculation of BRO cells. This subline possesses a higher metastatic potential than the parental BRO cell line. M14 [17] was a gift from Dr A. Cochran (John Wayne Clinic, Johnsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA). IF6 was developed in our laboratory from a melanoma metastasis in an axillary lymph node of a 35-year-old male patient (unpublished data). Mel 57 [5] was developed by Dr J. Brüggen (Münster, Germany) and was kindly provided by Dr J. de Vries (The Netherlands Cancer Institute, Amsterdam). 530 [38] was kindly provided by Dr P. I. Schrier (University Hospital, Leiden, The Netherlands). MV3 was derived from a cutaneous melanoma metastasis of a 76-year-old male patient after three cycles of *in vivo* passaging in nude mice [35].

All cell lines were grown as monolayers in culture flasks on Dulbecco's

modified Eagle medium supplemented with 10% fetal calf serum, glutamine, penicillin G and streptomycin.

Preparation of tumor cell suspension for in vivo *injections*

Tumor cells were harvested from subconfluent cultures by overlaying of the monolayers with a solution of 0·25% trypsin and 0·02% EDTA. After about 2 min the flasks were tapped sharply to dislodge the cells. Cells were washed twice with serum containing culture medium and then suspended in the appropriate concentration in phosphate-buffered saline (PBS).

Tumor cell injections

To produce xenografts at the inoculation site and to produce pulmonary metastases (spontaneous metastases) mice were inoculated s.c. with $1-2 \times 10^6$ tumor cells in a volume of 0·1 ml in the lateral thoracic wall [20]. The mice were inspected twice a week for local tumor growth and general condition. The number of s.c. injected mice ranged from 25 (MV3) to 60 (BLM).

To produce pulmonary tumor colonies (experimental metastases) $1-2 \times 10^6$ tumor cells in a volume of 0·1 ml were injected i.v. into the lateral tail vein. The number of i.v. injected mice ranged from 15 (530) to 45 (M14).

After s.c. and after i.v. inoculation mice were killed when signs of illness or respiratory distress were noted. Mice that remained healthy were killed 3–4 months after inoculation.

Detection of lung metastases

At autopsy all visceral organs were inspected. The tumor at the inoculation site, the lungs, spleen, liver and kidney and lymph nodes were taken for histopathological examination. Tissue fragments were: (a) fixed in 4% neutral formalin and embedded in paraffin for microscopic examination of hematoxylin and eosin stained 4 μm sections or (b) snap-frozen and stored at $-70°C$ for immunohistochemical analysis. As micrometastases can be missed by macroscopic examination only, paraffin sections from at least three different levels of the lungs were microscopically examined. From the other organs one representative section was examined.

Monoclonal antibodies

MAbs recognizing different MAAs or HLA antigens were used. Their antigenic specificity, their presumed role in melanoma biology and their source are listed in Table 1. All MAbs were purified from ascites fluid by affinity chromatography using a protein A–Sepharose column (Pharmacia, Uppsala, Sweden). All MAbs except anti-EGFR (2E9) were directly conjugated to horseradish peroxidase (HRPO) as described by Nakane and Pierce [27].

Immunocytochemical staining

To determine the immunocytochemical staining profile of the cell lines *in vitro* incubations were performed both on cytospin preparations and on cells grown on cover slips. Immunoperoxidase staining of xenograft lesions was performed on 4 μm frozen sections. The staining procedure was as follows: after acetone fixation preparations were incubated with directly HRPO-labeled MAb

Table 1.   Monoclonal antibodies used in this study.

Monoclonal antibody	Antigen	Biological role	Reference
NKI-beteb	gp100–107	MAA differentiation	[37]
K1.2	?	MAA progression	[32]
A1.43	Integrin VLA-2	MAA progression Cell–matrix, cell–cell adhesion	[4, 19]
Muc 18	gp113 Ig-superfamily	MAA progression	[21, 22]
15.75	gp75	MAA progression	[15]
PAL-M1	Transferrin receptor	MAA progression Cellular proliferation	[30, 36]
CL203.4	ICAM-1	MAA progression Adhesion	[26, 33]
B8.11.2	HLA-DR	Immune regulation	[23]
W6.32	HLA-ABC	Immune regulation	[2]
NKI-M9	MACAM-1	Cellular adhesion	[7]
PAL-M2	p95–100	Cellular adhesion	[30]
2E9	EGFR	MAA progression	[6, 8]

in an appropriate dilution for 1 h at room temperature. After washing they were stained using 3-amino-9-ethylcarbazole as chromogen. Preparations were counterstained with Harris hematoxylin and mounted for microscopic inspection. As negative controls identical incubations were done with an irrelevant HRPO-labeled MAb. Cytospin preparations and cover slips of all cell lines were also stained using an indirect immunoperoxidase technique. Xenografts were not stained by the indirect immunoperoxidase technique, as the binding of rabbit anti-mouse HRPO to the mouse tissue caused a strong aspecific signal that interfered with the interpretation of the antigen expression on the tumor cells.

Percentages of positive cells were estimated by two independent observers and expressed in classes indicating the percentage of positive cells: 0 (no positive cells), 1–5 (max. 5% positive cells), 6–20, 21–40, 41–60, 61–80, 81–100.

Results

Tumor take and metastasis rate

All cell lines used developed tumors at the s.c. inoculation site (Figure 1). Tumor growth after s.c. inoculation was most rapid with MV3 cells (tumor appearance after 10 days) and slowest with 530 cells (tumor appearance after 40 days). After 3–4 months tumor size in the 530 cell line inoculated mice was small (i.e. 10 mm diameter). Subcutaneous inoculation of the 530 cell line did not result in spontaneous metastasis. Even 6–7 months after s.c. inoculation lung metastases could not be found (not shown). After i.v. inoculation of 530 cells in 80% of the mice lung colonies were found (Figure 2A). M14, Mel 57, BLM (Figure 2B), and MV3 not only produced experimental metastases but also spontaneous metastases in 5, 15, 50 and 90% of the cases, respectively. No metastases were found in any of the other tissues examined. In Figure 3 the mean values of the number of lung metastases or lung colonies per three lung sections (see Materials and methods) is shown. In general, lung colonies from 530 cells are numerous and are small in size (Figure 2C) while the other cell
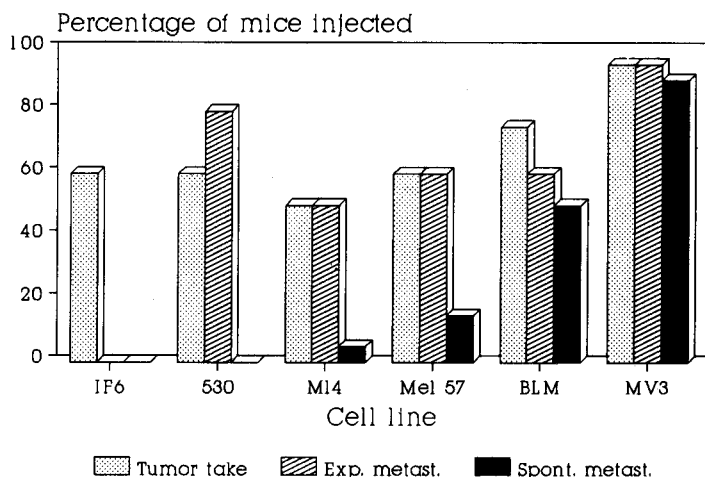
Percentage of mice injected

Figure 1. Diagram representing the percentage of tumor take and the metastasis rate of melanoma cell lines studied.

lines developed a relatively small number of lesions in the lungs; however, these lesions are larger in size.

Comparison of antigen expression in vitro *and in s.c. xenograft lesions*

To determine the expression of MAAs and of HLA class I and II antigens the staining profile of all six cell lines and their xenografted s.c. lesions (= inoculate) was studied. Antigen expression of each cell line both *in vitro* and in xenograft lesions was tested at least three times. Detailed positive staining results of all cell lines and their xenografts are summarized in Figure 4. MAbs that showed negative staining results with both the cell line and the xenograft lesions from a particular cell line are not depicted in the figure.

In s.c. xenograft lesions expression of the melanoma-associated differentiation marker NKI-beteb, of most progression and functional antigens, and of HLA class I was in general similar to the expression in the cell lines *in vitro* (Figure 4). Marked differences in expression between the cell lines *in vitro* and their s.c. xenograft lesions were seen with respect to HLA class II (B.8.11.2) and ICAM-1 (CL203.4). HLA class II antigens were detected on all cell lines in a moderate to high percentage of cells, except the M14 cell line where only a few cells were positive and the BLM and MV3 cell lines which were completely negative. In xenografts of all cell lines, except the 530 cell line, expression of HLA class II was not detectable in any of the lesions. In 530 tumors grown at the inoculation site 10–40% of the tumor cells were positive showing a diffuse distribution pattern.

ICAM-1 expression was found on all cell lines in a high percentage of cells. In xenografts of all cell lines ICAM-1 expression was not detectable, indicating a complete down-regulation of ICAM-1 expression.

Less striking but also remarkable was the difference in expression of NKI-beteb in the 530 xenograft lesions in comparison with the cultured cell line. While *in vitro* only very few cells were positive, in xenografts collected from the s.c. inoculation site nests of strongly positive cells were seen while only a small part of the surrounding tumor cells was stained (Figure 5A).
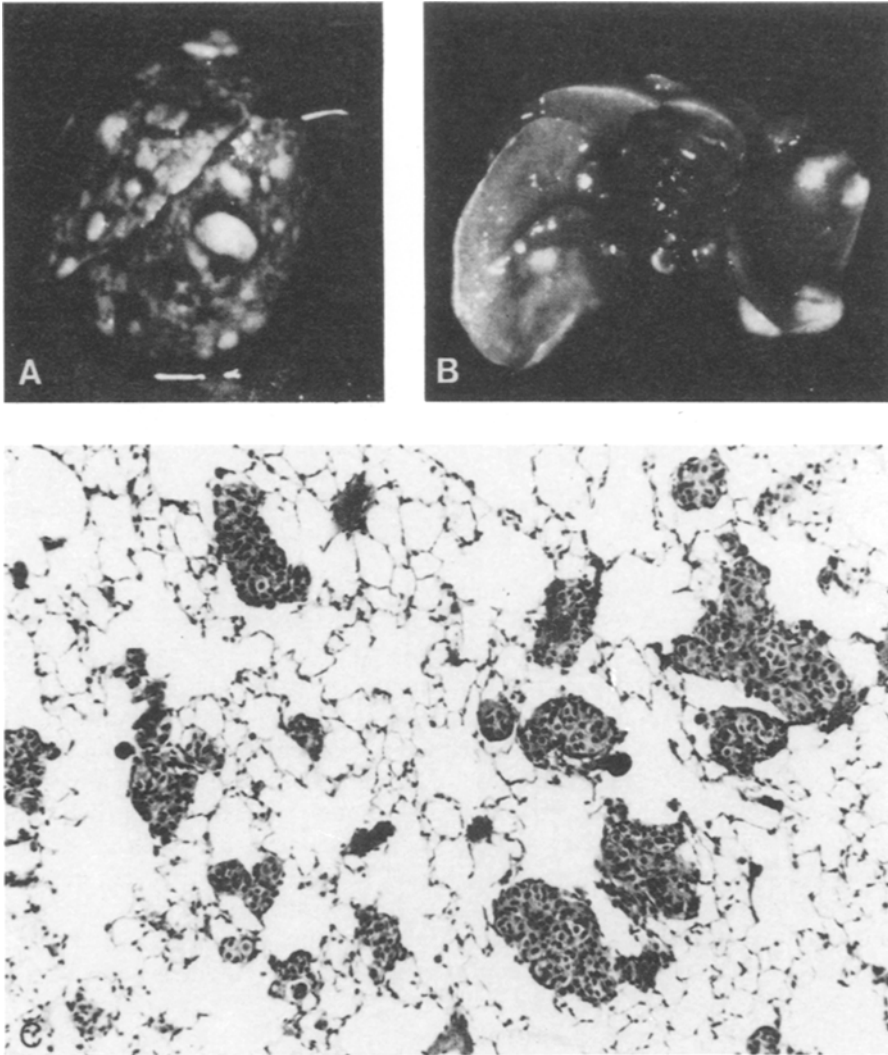
Figure 2. Lung metastases after inoculation of human melanoma cells. (A) Macroscopic preparation of the lungs from a mouse i.v. inoculated with 530 cells. Note the numerous lung colonies. (B) Macroscopic preparation of the lungs from a mouse s.c. inoculated with BLM cells. Note the tumor cell nodules which are visible on the lung surface. (C) Microscopic section from the lungs shown in (A). ×160.

Finally, marked expression of epidermal growth factor receptor (EGFR) (not shown in Figure 4) was seen on BLM and MV3 cells *in vitro* while all other cell lines were negative. Only M14 cells showed very faint staining. No xenograft lesions were stained with anti-EGFR due to absence of a directly labeled antibody.

Comparison of antigen expression between s.c. tumors and lung metastases
To determine whether any of the antigens studied may be specifically expressed as a consequence of the metastasizing process we also compared antigen expression of s.c. tumors and lung metastases, both experimental and
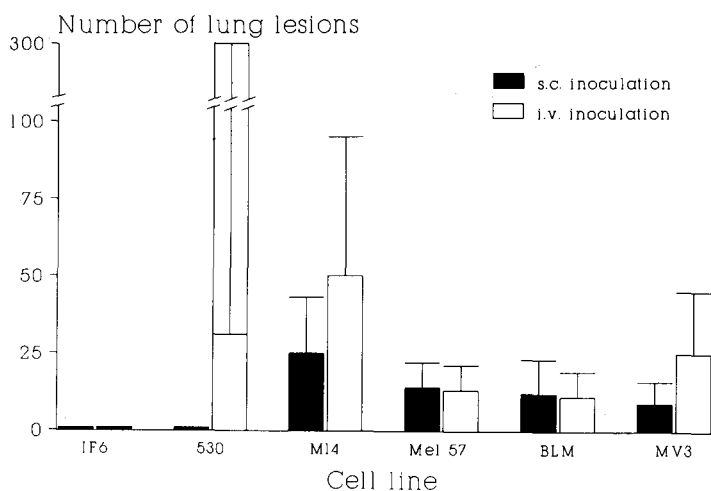
Figure 3. Diagram representing the mean value of the number of lung lesions counted in three lung sections from three different levels of the lungs.

spontaneous. Except the 530 cell line, all cell lines that gave rise to lung colonization (i.v. inoculation) and/or lung metastasis (s.c. inoculation) showed (nearly) identical expression patterns in s.c. tumors and lung lesions (Figure 4). With respect to the 530 cell line we found that expression of some antigens (e.g. NKI-beteb, NKI-M9) was variable. Although expression in s.c. tumors was rather constant, in the lungs colonies were seen which were either completely negative, partly positive or completely positive, often very strongly (Figure 5B).

As in s.c. tumors, lung lesions of all cell lines except 530 showed strong or complete down-regulation of both HLA class II and ICAM-1 expression. In lung colonies of the 530 cell line expression of HLA class II was absent in most lesions. A few lesions showed only scattered weakly positive cells. However, we found one very strongly positive tumor lesion localized at the visceral pleura (Figure 5C).

A remarkable difference in expression of Muc 18 antigen between the s.c. tumor and the lung lesions was seen with Mel 57 cells. While s.c. tumors and lung colonies were clearly positive spontaneous metastases showed positive as well as negative lesions.

Comparison of metastasizing and non-metastasizing cell lines

The data presented in Figure 1 indicate that we used a panel of human melanoma cell lines with different biological behavior in nude mice, especially with different capacities to form spontaneous lung metastases: IF6 and 530 do not produce spontaneous lung metastases, M14 and Mel 57 only sporadically, and BLM and MV3 frequently. When we evaluate the potency of the cell lines to form spontaneous metastases and their immunophenotype *in vitro* we see that absence of NKI-beteb and HLA class II and marked expression of EGFR and VLA-2 antigen (BLM and MV3) is associated with a high capacity to form spontaneous metastases (Table 2). An inverse expression pattern of these antigens *in vitro* is associated with no or very low metastatic ability (IF6, 530, M14 and Mel 57).
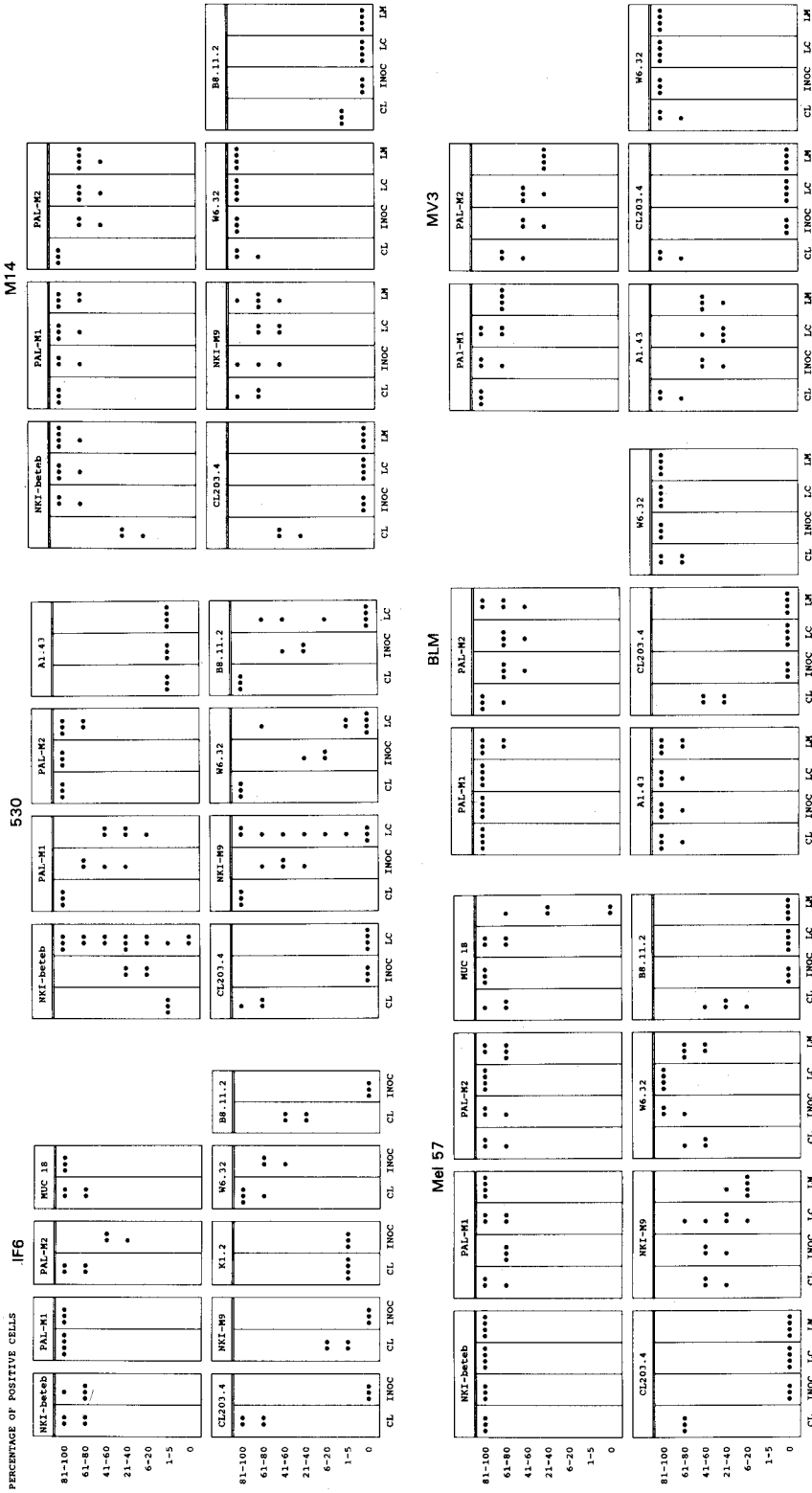
Figure 4. Percentage of positive melanoma cells in the cultured cell line (CL), in the tumor at the s.c. inoculation site (INOC), and in the lung lesions after i.v. (LC) and s.c. (LM) tumor cell administration. CL, cell line; INOC, inoculate; LC, lung colony; LM, lung metastasis.
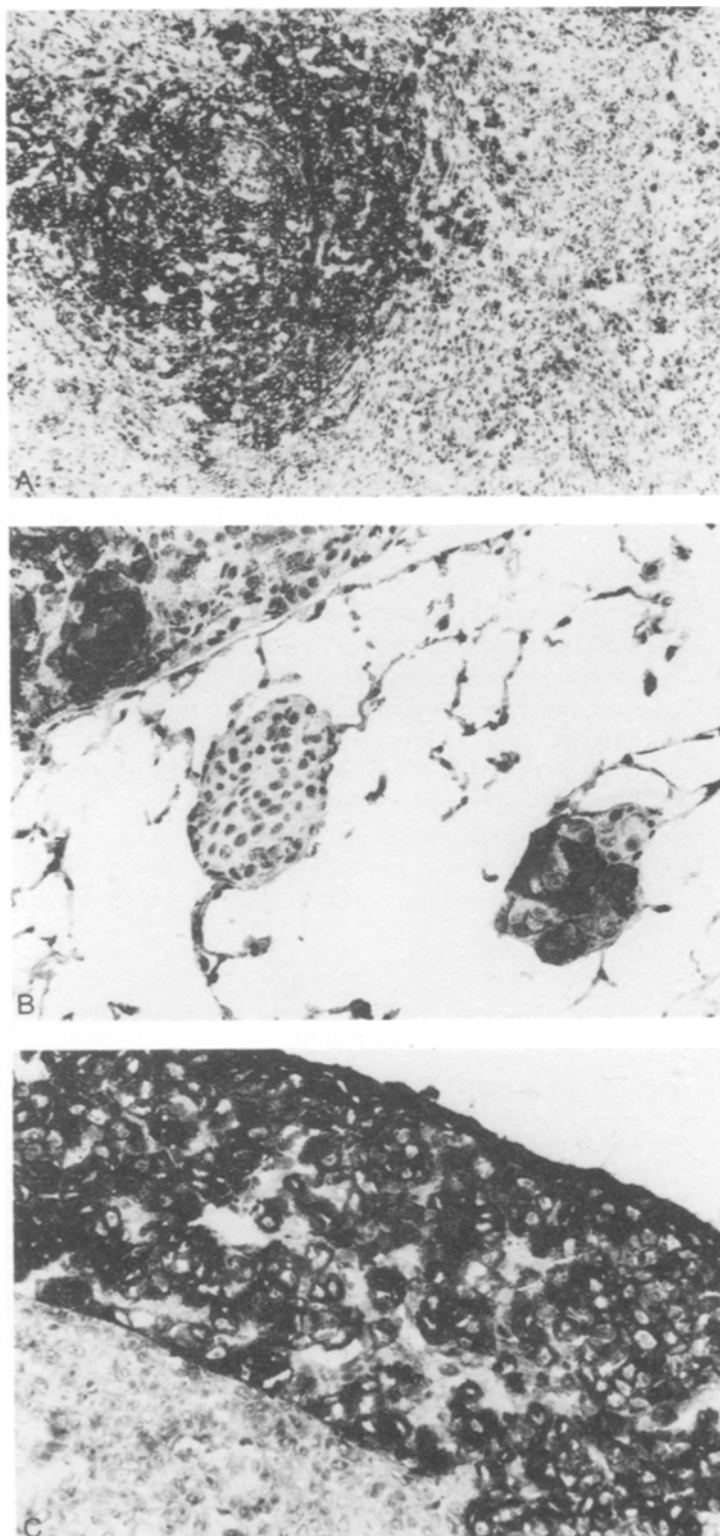
Figure 5.   Tumor growing at the inoculation site after s.c. injection of 530 cells (A) and lung colonies developed after i.v. inoculation of 530 cells (B, C). Frozen sections are stained with NKI-beteb (A, B) and anti-HLA class II (C). (A) ×128; (B, C) ×320.

Discussion

In this study we used a panel of human melanoma cell lines with different biological behavior in nude mice ranging from non-metastatic (IF6), moderate metastatic (530, M14 and Mel 57) to highly metastatic (BLM and MV3).

Our immunocytochemical results show that the nude mouse provides a good model to study the phenotype of metastasizing or colonizing human tumor cells in subsequent stages of the process of dissemination in one individual. With respect to the expression of the antigens studied we found that the expression pattern in xenograft lesions was in general similar to the expression on the cell lines *in vitro*. We found only few exceptions; the most striking being the lack of HLA-DR antigen expression in xenografted lesions produced after s.c. as well as i.v. inoculation of all cell lines but one, the 530 cell line.

That this phenomenon is selective is strongly suggested by the fact that HLA class I and most MAAs tested showed a staining pattern in all xenografts that was in general similar to that in the melanoma cell lines *in vitro*. The lack of HLA class II expression could be explained by clonal outgrowth of class II negative cells. However, this seems unlikely because the growth behavior of the class II negative BLM cell line showed no large differences with other, class II positive, cell lines. In addition, expression of HLA-DR can be reinduced by culturing melanoma cells derived from HLA-DR negative xenograft lesions (own observation). Another explanation for the dramatical down-regulation in the xenografts may be the presence of some factors in the tumor environment or the lack of human inducing factors, e.g. IFN-gamma [13]. Recent evidence has shown that lymphokines such as IFN-gamma induce the expression of HLA class II antigens in cells of the melanocytic lineage [9, 11, 14, 25, 28]. In addition, recently it was shown that *in vivo* administration of recombinant IFN-gamma to nude mice bearing human tumor xenografts can result in an induction of HLA-DR expression on the tumor cells ([1], own observation). A third

Table 2. **Relation between biological behavior of human melanoma cell lines in nude mice and expression of MAAs.**

Cell line	Lung metastasis		Expression of MAAs			
	Exp. (%)	Spont. (%)	HLA-DR	EGFR	VLA-2	NKI-beteb
IF6	0	0	+ (30–50)	–	–	+ (70–90)
530	80	0	+ (80–100)	–	+/– (1–5)	+ (1–5)
M14	50	5	+ (1–5)	+/– (80–100)	–	+ (20–40)
Mel 57	60	15	+ (20–40)	–	–	+ (80–100)
BLM	60	50	–	++ (80–100)	++ (80–100)	–
MV3	100	90	–	++ (80–100)	++ (80–100)	–

–, Negative staining; +/–, very weak staining; +, moderate staining; ++, strong staining. Percentage of tumor cells stained is shown in parentheses.

explanation may be that the presence of human melanoma cells in nude mice may give rise to a change in the differentiation stage of the melanoma cells. However, concerning the melanoma cell lines studied no changes in stage of differentiation in xenografts could be detected. The results with the 530 cell line indicate that lack of expression of class II in the xenografts of the other cell lines is not a complete switch-off of the HLA class II genes, but suggest that there is a dramatic down-regulation of the expression. The expression of HLA class II in part of the 530 cells in xenograft lesions may be caused by constitutive expression of the gene in these cells instead of an inducible expression in the other cells. A down-regulation as seen for HLA-DR was also found for ICAM-1 in xenograft lesions. Previous studies as well as our own observations (not published) have shown that on melanocytic cells expression of ICAM-1, just as HLA-DR, is susceptible to modulation by IFN-gamma [26, 33].

Mainly based on the marked higher expression of ICAM-1 in melanoma metastases than in primary melanomas it was suggested that ICAM-1 could have a functional role in the process of dissemination [16, 25]. However, in our animal model system we did not find an induction of ICAM-1 expression in metastatic lesions indicating that in this system there seems no important role for this adhesion molecule in metastasis. In addition, if ICAM-1 plays a role in metastasis formation in nude mice, human ICAM-1 should recognize the active site of its counter structure, mouse LFA-1. To the best of our knowledge until now no data are available with respect to this cross-reactivity.

In all melanoma cell lines studied except 530, the degree of intra- and interlesional heterogeneity regarding the expression of MAAs and HLA antigens is limited. This is in line with recent data showing that there is a limited heterogeneity within fresh human melanoma lesions [8], which may reflect clonal overgrowth [18]. The intra- and interlesional heterogeneity of 530 xenograft tumors may be explained by the fact that this cell line has only recently been developed.

In a recent study Rodolfo *et al.* [29] reported data on the growth behavior of early cell cultures of different primary and metastatic melanomas in nude mice. In that study they also described the tumor cell phenotype of these early cultures between the third and eighth *in vitro* culture generation for HLA class I and II and some MAAs using an indirect immunodetection technique. With respect to the expression of melanocytic differentiation markers, HLA-ABC, HLA-DR and ICAM-1 *in vitro* their results are in agreement with ours.

Our experiments suggest that there is a striking difference in expression of the progression markers VLA-2, EGFR and HLA class II and of the differentiation antigen NKI-beteb *in vitro* between cell lines with different biological behavior in nude mice. Expression of the first two antigens and lack of expression of the latter two is associated with the capacity to form spontaneous lung metastases. However lack of expression of HLA class II does not seem to play a crucial role in the biological behavior of the tumor cells in the *in vivo* model system because expression of this antigen is completely switched-off or dramatically down-regulated in all xenograft lesions. Whether the expression of VLA-2 and EGFR and lack of expression of NKI-beteb are really involved in spontaneous metastasis formation has to be proven. With respect to the intracellular antigen NKI-beteb we suppose that lack of expression in BLM and MV3 has been caused by loss of differentiation of the melanoma cells.

The melanoma-associated progression antigens identified so far have been defined by the correlation of their expression with human melanocytic tumor progression and in part (e.g. HLA class II) for their ability to predict the appearance of metastases or unfavorable outcome [3, 34]. This implicates that these markers are candidates for molecules which may play a role in the pathogenesis of metastasis in human melanoma. Our study showed that cell lines (BLM and MV3) with high metastatic capacity express few of the markers of tumor progression while the less aggressive cell line (IF6) expresses most of these antigens. This negative correlation between the number of progression markers expressed and the degree of malignancy is not in line with the correlation found in the human melanocytic system. Therefore, it is not certain if these markers play a role in the process of metastasis. It is conceivable that they are involved in clonal expansion. Furthermore, as already pointed out for ICAM-1, all of the interactions which should occur between the tumor cells and other cells or tissue structures, in metastasis formation of human tumors in the nude mouse are heterologous. Therefore, whatever interactions are occurring, only those where ligand–ligand interactions are well conserved between these species may be relevant for the human situation. These interactions may be mainly limited to cell–substrate interactions (e.g. EGF and EGFR; extracellular matrix components and their receptor molecules) rather than cell–cell interactions. With respect to our data this means that only some of the antigens studied, e.g. EGFR and VLA-2, may have relevance in the human situation. Further studies using transfected human melanoma cells are in progress to obtain more evidence for the role of these gene products in the pathogenesis of metastasis.

Acknowledgements

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