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Isolation and Visualization of Met-72-Positive, Metastatic Variants Present in B16 Melanoma Tumor Masses

Nanette P. Parratto and Arthur K. Kimura

Department of Pathology, University of Florida College of Medicine, Gainesville, Florida 32610

Metastatic variants of the B16 melanoma displaying high experimental metastatic potential have been shown to express high levels of a 72,000-dalton glycoprotein (Met-72) on their cell surface (Kimura AK, Xiang J: *J Nat Can Inst* 76:1247-1253, 1986). Monoclonal antibodies (MoAb) directed against the Met-72 determinant have been used in this study as immunohistochemical reagents on preparations of fresh B16 melanoma tumors and their metastases. These immunohistochemical analyses have utilized frozen sections, impression smears, and cytospin preparations of fresh tumors harvested at various time points during tumor growth, to view the presence and location of Met-72-positive metastatic variants within tumor masses. Biotinylated anti-Met-72 MoAbs were reacted with freshly dissociated tumor cells from a B16 melanoma ovarian metastasis. These cells were then reacted with fluorescein isothiocyanate (FITC)-streptavidin and analyzed by flow cytometry. A discrete population of positively staining cells was detected and isolated by cell sorting techniques. Met-72-positive cells were then cloned and reanalyzed after several weeks of *in vitro* expansion and found to have high experimental metastatic potential to ovaries. Frozen sections of subcutaneous tumors and their metastases were analyzed by immunoperoxidase techniques. A consistent finding in these studies has been that the few tumor cells which showed high intensity of Met-72 staining were positioned perivascularly and at the invading front of B16 melanoma tumors.

Key words: B16 melanoma, metastatic variants, met 72/83 antigen, immunohistochemistry, localization *in situ*

A number of experimental systems have documented clonal heterogeneity within primary tumors and their metastases [1-5]. Thus, although certain tumors may be

Abbreviations used: ABC = avidin-biotin-horseradish peroxidase complex; AEC = amino ethyl carbazole; cPEG = phosphate buffered saline + ethylenediamine-tetraacetic acid + glucose; FACS = fluorescence activated cell sorter; FBS = fetal bovine serum; FITC = fluorescein isothiocyanate; MoAb = monoclonal antibody (ies); NCS = newborn calf serum; pA = protein A; PBS = phosphate buffered saline; sA = streptavidin.

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judged clonal with respect to a given phenotype or cell surface marker, clonal components of the tumor can differ markedly in their ability to form metastases, or in susceptibility to immune attack or drug therapy [2–5]. Tumor cells with high metastatic potential have often been referred to as metastatic variants.

Histopathologic evaluation of most solid tumors has been hindered by the lack of reagents capable of specifically identifying metastatic variants. Direct, *in situ* visualization of metastatic tumor cell variants would provide a valuable handle towards understanding the clonal evolution and interactions of tumor cells within primary tumors and their metastases. Recently, we identified via monoclonal antibody (MoAb), a 72-kilodalton (Kd) cell surface glycoprotein (Met-72) quantitatively associated with highly metastatic tumor cell variants of the B16 melanoma [1]. The experimental metastatic potential of over 30 B16 melanoma clones has been correlated to a quantitative surface expression of Met-72 [1,6,7]. In addition, anti-Met-72 MoAb used in conjunction with fluorescence-activated cell sorting (FACS) has recently been used directly to isolate metastatic variants from the heterogeneous parental B16-F1 tumor [8].

The present study was designed to isolate and histologically localize Met-72-positive, metastatic variants present in fresh B16 melanoma tumor masses. Perhaps the most striking finding to emerge from these studies was the unique, localized distribution of Met-72-positive cells within the tumor mass. Anti-Met-72 MoAb staining was only observed on tumor cells located perivascularly and along the invading front of the developing tumor. These experiments show that our MoAb previously used to characterize the expression of Met-72 *in vitro*, may also be useful for isolation and localization of highly metastatic variants *in situ* in primary and metastatic B16 melanoma.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from the Jackson Laboratory, (Bar Harbor, ME) and housed in the Tumor Biology Unit mouse colony, Department of Pathology. Female mice, aged 8–16 weeks, were used in these studies.

Monoclonal Antibodies

Anti-Met 72 MoAb were generated by syngeneic immunization of C57BL/6 mice with selected B16 melanoma clones. The specificity and characteristics of these MoAbs have been reported in detail [1]. Hybridoma cells secreting an isotype-identical, negative control MoAb used in this study (anti-sheep red blood cell, N- S.7, IgG3: kappa) were obtained from the American Type Culture Collection (Rockville, MD).

Murine Melanoma Cell Lines

The C57BL/6 melanoma, B16, and various *in vitro* and *in vivo* selected lines derived from it were obtained from the Division of Cancer Treatment Tumor Bank (E.G. and G. Mason Research Institute, 57 Union St., Worcester, MA) where they had been deposited by Dr. I.J. Fidler. Clones were derived by limiting dilution and micromanipulation [1] from both the parent line, B16-F1, and the *in vitro* selected, highly invasive metastatic form, B16-BL6 [9]. Stocks from early passages of these

lines and clones were frozen at −70°C and restarted every 8–12 weeks to limit the possibilities of functional and phenotypic drift. All cell lines and clones were maintained *in vitro* at 37°C in a humidified incubator containing 8% CO_2, by subculturing every 4 days. Monolayers of cells were detached from the petri dishes (Costar #3100, Cambridge, MA) by a 3-min room-temperature incubation with 0.5 mM EDTA in Ca^{++}- and Mg^{++}- free phosphate buffered saline (PBS) supplemented with 0.1% glucose (cPEG) [10]. For routine passage, cells were washed and replated at a concentration of 5×10^5/10 cm dish in 10 ml media.

The parent melanoma line, B16-F1, was maintained in Cellgro MEM (Sybron, Washington D.C.) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml), 1 mM pyruvate, 2 mM glutamine, and 0.225% $NaHCO_3$. The highly invasive metastatic clone, BL6-10, was maintained in Eagles' Hanks' amino acid supplemented medium (EHAA, GIBCO, Grand Island, NY) [11] containing 10% newborn calf serum (NCS), penicillin, and streptomycin. A recently derived, poorly metastatic C5BL/6 melanoma, *JB/RH*, was provided by Dr. Jane Berklehammer (AMC Cancer Research Center, Denver, CO) [12,13] for comparison in our studies. These cells were maintained and passaged *in vitro* in EHAA + 10% NCS.

Biotinylation of MoAb and Flow Cytometric Analysis

IgG was purified from culture supernatants or ascites fluid by affinity fractionation through protein A-Sepharose 4B (Pharmacia, Piscataway, NJ) [14] and checked for purity by SDS polyacrylamide gel electrophoresis [15]. Affinity-purified IgG (1 mg/ml) was dialyzed against 0.1 M $NaHCO_3$ (pH 8.2) and then reacted with 2 mM NHS-LC-biotin (Pierce Chemical, Rockford, IL) dissolved in dimethylformamide (Sigma, St. Louis, MO) at biotin ester:protein ratios of 1:2.5 to 1:10 (w/w) for 4 hr at room temperature, in the dark. The reaction was stopped by the addition of 1M NH_4Cl to a final concentration of 0.1 M in the reaction mixture. Unreacted biotin was then removed by exhaustive dialysis against PBS. Biotin-conjugated protein concentrations were determined by optical density at 280 nm [16]. Optimal biotin ester/protein ratios used for the conjugation of the various preparations were determined empirically by flow cytometric analysis on cell preparations [17]. Briefly, 2×10^6 melanoma cells were incubated with biotinylated antibody at 4°C for 30 min in the dark. After three washes in PBS with 5% agamma horse serum (GIBCO), either native or biotinylated MoAb was incubated with FITC-streptavidin (Zymed Laboratories, San Francisco, CA) or FITC-sheep antimouse Ig for nonbiotinylated preparations. After three washes, 2×10^4 cells were counted and analyzed by flow cytometry [8,17].

Measurement of Purified and Biotinylated MoAb Binding by Radioimmunoassay

MoAb binding to the various cell types was measured indirectly with ^{125}I protein A (pA) as described [18] or with ^{125}I streptavidin (sA) with biotinylated MoAb using a modification of the method of Philpott et al [19]. Briefly, 2×10^5 freshly harvested *in vitro* grown cells were incubated for 1 hr with various dilutions of purified native or biotinylated MoAb. The cells were then washed three times with PBS plus 5% agamma horse serum and incubated for an additional 1 hr with 2×10^5 cpm radiolabeled pA or sA. Radioactivity bound to the cells after three washings was

assessed by gamma scintillation counting. Background binding in these assays was routinely less than 600 cpm. MoAb binding to the different cell types is expressed as a binding index [1], calculated from triplicate determinations as follows:

$$\text{Binding index} = \frac{\text{mean cpm bound with anti-Met-72 MoAb}}{\text{mean cpm bound with anti-sheep red blood cell MoAb}}$$

This normalizes individual differences in background binding and allows comparison between the different cell types.

Generation of Subcutaneous Primary Tumor Foci, Spontaneous Metastases and Lung Colonization Assay

Primary tumor masses were generated by s.c. injection of 2×10^6 cells/100 μl PBS, at the dorsal thoracolumbar spinous junction. Experimental metastases were generated by tail vein injection of 3×10^5 cells into age- and sex-matched C57BL/6 mice.

Spontaneous metastases were generated by subcutaneous inoculation of 2×10^6 cells followed in 10 days by tumor excision with necropsy 21 days later (31 days after primary subcutaneous injection). Tumors were excised at various points of tumor growth.

Immunocytochemistry of Cytospin and Impression Smear Preparations of B16 Melanoma

B16 melanoma tumors were brought to a single cell suspension by teasing tumor masses into approximately 1 mm^3 pieces which were then subjected to an 8–10-hr incubation in Eagle's media supplemented with 0.1% collagenase V (Sigma, 10 ml/g tumor weight) at 4°C [20]. Viable cells were separated on Ficoll Hypaque (Pharmacia, Sweden) and resuspended in PBS. Cells were incubated with 0.3% H_2O_2 in PBS plus 5% agamma horse serum for 30 min at 4°C and washed three times with PBS. The cells were then incubated with biotinylated MoAb for 30 min at 4°C in the dark and washed three times. The antibody-labeled cells were incubated with avidin-biotin-horseradish peroxidase complex ([ABC]) Vector Laboratories, Burlingame, CA) for 30 min at 4°C in the dark, and washed three times. Approximately 3×10^4 labeled cells were then cytocentrifuged onto subbed slides. Slides were fixed in acetone for 30 sec, washed in PBS, and the reaction developed with aminoethylcarbazole ([AEC] Sigma) in the presence of H_2O_2, washed and counterstained with hematoxylin [21].

Impression smears of excised tumors were made by gently touching tumors, prerinsed in ice-cold PBS, onto subbed slides. The slides were air dried, fixed in acetone for 10 min, rinsed in PBS, and dried. Slides were either processed by immunohistochemistry methodology (see below) or stored at −70°C for future use. Impression smears provide a rapid screening method for detection of antigen expression *in vivo* [22].

Immunohistochemistry of Cryostat Sections of Primary and Metastatic B16 Melanoma

Primary and metastatic tumor masses obtained after 5–21 days of growth were excised, snap frozen in isopentane, and liquid nitrogen and stored at −70°C until

sectioned. Cryostat sections 4 μm thick were air dried, fixed in cold acetone for 10 min, and washed in PBS using a modification of the technique by Suter et al [23]. Endogenous peroxidase was blocked by reaction with 0.3% H_2O_2 in PBS plus 5% agamma horse serum for 30 min at room temperature. Biotinylated antibodies at predetermined optimal concentrations were layered onto sections and allowed to react for 60 min at room temperature in a dark humidified chamber. Slides were then washed in three changes of PBS and reacted with ABC reagent for 30 min at room temperature in a dark, humidified chamber, and the slides were then washed in three changes of PBS. The reaction was developed with AEC in the presence of H_2O_2. The slides were washed, counterstained with hematoxylin, and examined under oil immersion.

RESULTS

Biotinylated Anti-Med-72 MoAb Specificity

Affinity purified anti-Met-72 or control MoAb were reacted with the biotin ester at varying weight/weight ratios. Flow cytometric analysis of biotinylated MoAb binding to B16 metastatic variants or sheep red blood cells was used to optimize conditions for biotin substitution. Three C57BL/6 melanoma lines of high and low experimental metastatic activities (Table I) were used as target cells in radioimmunoassay to insure that the process of biotinylation did not alter the specificity of anti-Met-72 MoAb. The ability of biotinylated anti-Met-72 MoAb to bind to murine melanomas of high metastatic potential is shown in Figure 1. Binding of biotinylated MoAb was measured indirectly after the addition of ^{125}I-streptavidin. The highly metastatic B16 melanoma clone, BL6-10, shows an approximately fivefold higher level of anti-Met-72 MoAb binding in RIA than to the poorly metastatic cell lines B16-F1 and *JB/RH*.

FACS Selection of Met-72-Positive Variants Isolated From a Fresh Experimental Ovarian Metastasis

Experimental metastases were generated by i.v. injection of B16-F1 melanoma cells as described. An ovarian metastasis was noted 21 days later, removed, and brought to a single-cell suspension for analysis of Met-72 expression by flow cytom-

TABLE I. Experimental Metastatic Potential of Three C57BL/6 Murine Melanomas*

Experiment	Cells	n[a]	Mean No. metastases pulmonary ± SE	Other sites of metastases
A	B16-F1	4	18 ± 3	Ovary, lymph node, liver
B	B16-F1	5	56 ± 16	Bone marrow
A	BL6-10	6	261 ± 96	Bone marrow, lymph node
B	BL6-10	14	160 ± 146	Ovary, bone marrow, lymph node, subcutis
A	JB/RH	6	0	
B	JB/RH	9	0	

*Experimental metastases were generated by tail-vein injection of 3×10^5 cells per 0.2 ml PBS.
[a]No. of age-matched female mice per group.

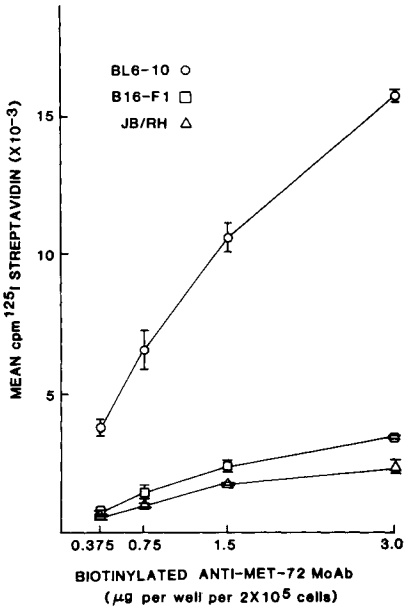

Fig. 1. Retention of binding specificity of anti-Met-72 MoAb after biotinylation. Various amounts of biotinylated anti-Met-72 MoAb were reacted with the highly metastatic B16 melanoma clone, BL6/10, the poorly metastatic B16 melanoma parent, B16-F1, and a recently derived, poorly metastatic C57BL/6 melanoma, *JB/RH*. The extent of specific binding was measured after the addition of ^{125}I-labeled streptavidin. Values are expressed as the mean of triplicate determinations of cpm bound ± SE.

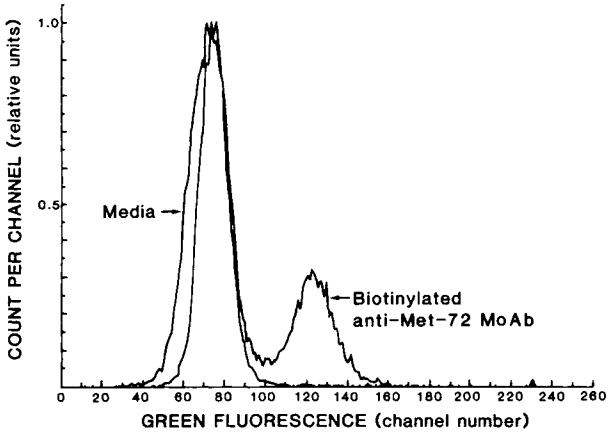

Fig. 2. Flow cytometric analysis of Met-72-positive metastatic variants within a fresh ovarian metastasis.

etry. Biotinylated anti-Met-72 MoAb and FITC-streptavidin treatment of these cells revealed two distinct populations within the freshly excised ovarian metastasis, one highly Met-72 positive (Fig. 2). Melanoma cells sorted from this population and maintained *in vitro* have retained a high binding profile to anti-Met-72 MoAb (data not shown). Cells obtained from the original ovarian metastasis (0-1) were cloned. One clone from the original ovarian metastasis was successively cycled by intravenous passage in the syngeneic host, three times (0-1.1, 0-1.2, 0-1.3), and each time organ

selectivity and Met-72 expression of ovarian metastases were recorded. Repeated passage *in vivo* was seen to enhance organ selectivity for ovaries (53%, 56%, 87% of the mice having ovarian metastases) and to enrich for Met-72 expression (28, 36, 45 times background) (Fig. 3).

Immunocytology of Fresh Ovarian and Lung Metastases

B16 melanoma metastatic variants were isolated from fresh lung and ovarian experimental metastases by mechanical and enzymatic disaggregation. Single-cell suspensions were processed for immunocytology using anti-Met-72 MoAb. Cytospin preparations of an experimental ovarian metastasis showed positive immunoperoxidase staining (Fig. 4B). Impression smears of experimental and spontaneous lung metastases generated by injection of the poorly metastatic F1 parental B16 melanoma demonstrated similar levels of individual cellular binding to anti-Met-72 MoAb (Fig. 4D,F). Isotype identical MoAb (anti-sheep RBC, N-S.7) showed no staining in these assays (Fig. 4A,C,E).

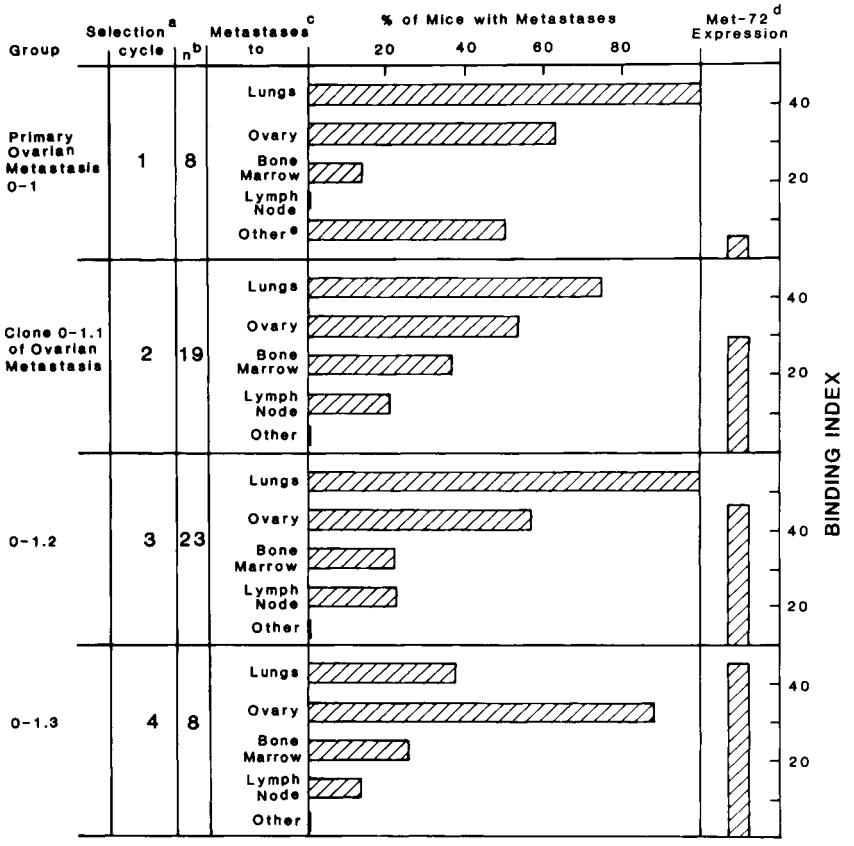

Fig. 3. Clones derived from experimental metastases to ovaries retain a high expression of Met-72 upon repeated cycling *in vivo*. **a:** In vivo cycle number for the selection of metastatic variants with ovary specificity. **b:** Number of mice per group. **c:** Organ site of metastasis. **d:** Binding of anti-Met-72 MoAb to ovarian metastatic variants as detected by RIA. Results are expressed as a binding index, which is calculated by dividing the mean cpm ^{125}I pA bound with anti-Met-72 MoAb divided by the mean cpm ^{125}I pA bound with N-S.7 MoAb (background binding). **e:** Other = kidney, mesentery.

Localized Distribution of Met-72-Positive Variants Within Progressing Subcutaneous Melanoma

Immunohistologic analyses of cryostat sections from subcutaneous B16-F1 melanomas were performed at various time points during tumor growth. Predetermine-doptimal concentrations of biotinylated MoAb were incubated with serial sections of snap-frozen tumor. Reactive sites were detected as red granules upon development with AEC. A common pattern of reactivity has been noted in all sections examined from tumors as early as 3 days to as late as 15 days of growth. A subcutaneous B16-F1 tumor excised after 7 days of growth was serially sectioned and stained with anti-Met-72 MoAb (Fig. 5). Background levels of peroxidase staining are shown in Figure 5A,B, using an isotype-identical biotinylated control MoAb, N-S.7. In contrast to the rather uniform staining of melanoma cells obtained from metastases (Fig. 4B,D,F), Met-72-positive cells seen in cryostat sections of primary tumors were observed only on advancing fronts and leading edges of the tumor mass (Fig. 5C,D,F), perivascular to extra-tumor vessels and intravascularly (Fig. 5E). No detectable binding was seen in the bulk of the tumor mass, which consisted of differentiated melanoma cells surrounding a necrotic, poorly vascularized, central core.

DISCUSSION

The existence of subpopulations of cells exhibiting a range of metastatic potential within heterogeneous tumors has been substantiated in a number of systems [2]. In the original studies leading to the present work, a strong correlation between the quantitative expression of a 72-Kd glycoprotein (Met-72) and experimental metastatic activity of over 30 *in vitro* growth B16 melanoma clones was demonstrated [1,6]. Flow cytometric analysis and cell sorting procedures using anti-Met-72 MoAb have directly shown that high levels of Met-72 expression are characteristic of cells with a high experimental metastatic potential [8]. Our current studies were designed to determine the potential utility of anti-Met-72 MoAb to visualize and localize Met-72-positive metastatic variants within progressively growing and metastatic B16 melanoma masses.

Results of the experiments reported here greatly expand our knowledge of Met-72 antigen expression and its correlation with metastatic potential *in vivo*. Localization of its expression in primary subcutaneous tumors is notably discrete, and not randomly distributed throughout the developing tumor mass.

Immunohistologic examination of progressively growing tumors excised at various times during subcutaneous growth shows a recurrent pattern of localization of Met-72-positive variants. In the present study we elected to study the staining and distribution of Met-72-positive, metastatic variants within the parent B16-F1 melanoma and metastases derived from it. Similar studies have been performed with our highly metastatic clone BL6-10, which was originally selected from the B16-F10 for increased invasiveness through bladder wall [9]. Subcutaneously derived tumors generated from both the parental B16-F1 and BL6-10 have been examined after 3, 7, 9, 12, and 15 days of growth. Met-72 expression was only observed on certain cells seen at the advancing front and perivascularly. These findings suggest that microenvironmental influences may function regionally to influence metastatic potential. The implication of this selective localization suggests that even if random somatic mutational events yield single metastatic variant cells within a solid tumor mass, their

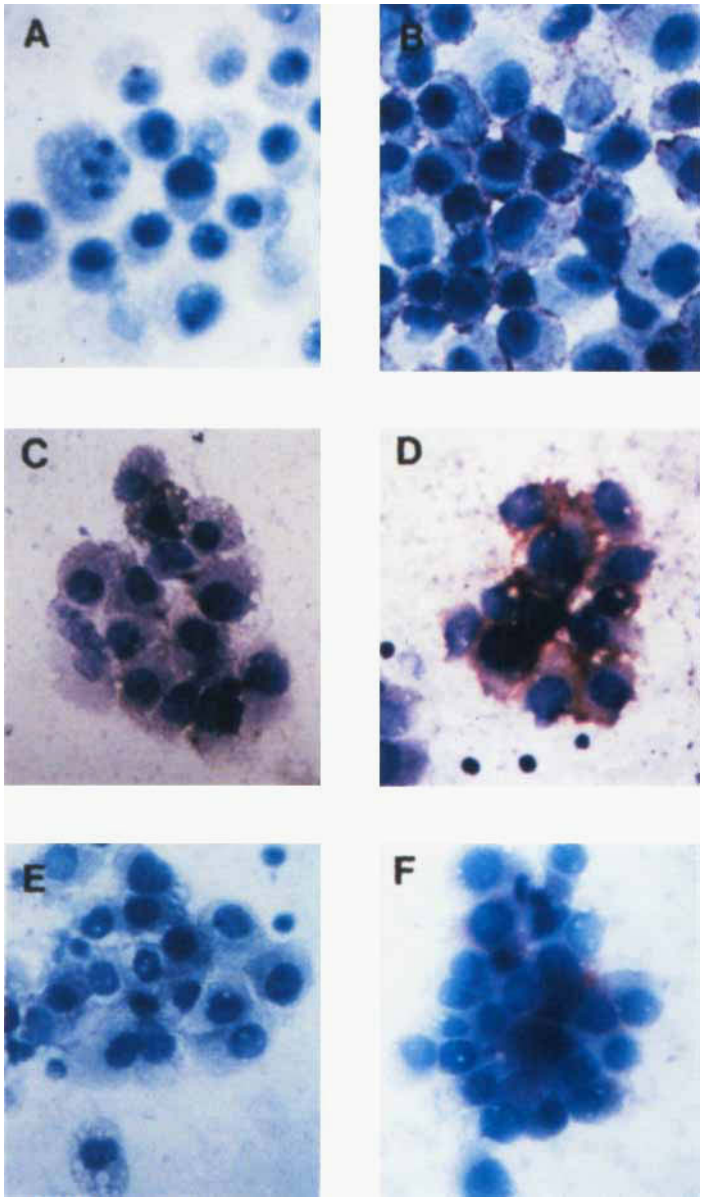

Fig. 4. Met-72-positive variants of B16 melanoma detected in cytospin and impression smear preparations of metastases. Cell suspensions from a fresh ovarian metastasis were stained using biotinylated control N-S.7 MoAb (**A**) or anti-Met-72 MoAb (**B**). Impression smears of experimental lung metastases (**C, D**) or spontaneous lung metastases (**E, F**) were stained using biotinylated N-S.7 MoAb (C,E) or anti-Met-72 MoAb (D,F). A–F, ×128.

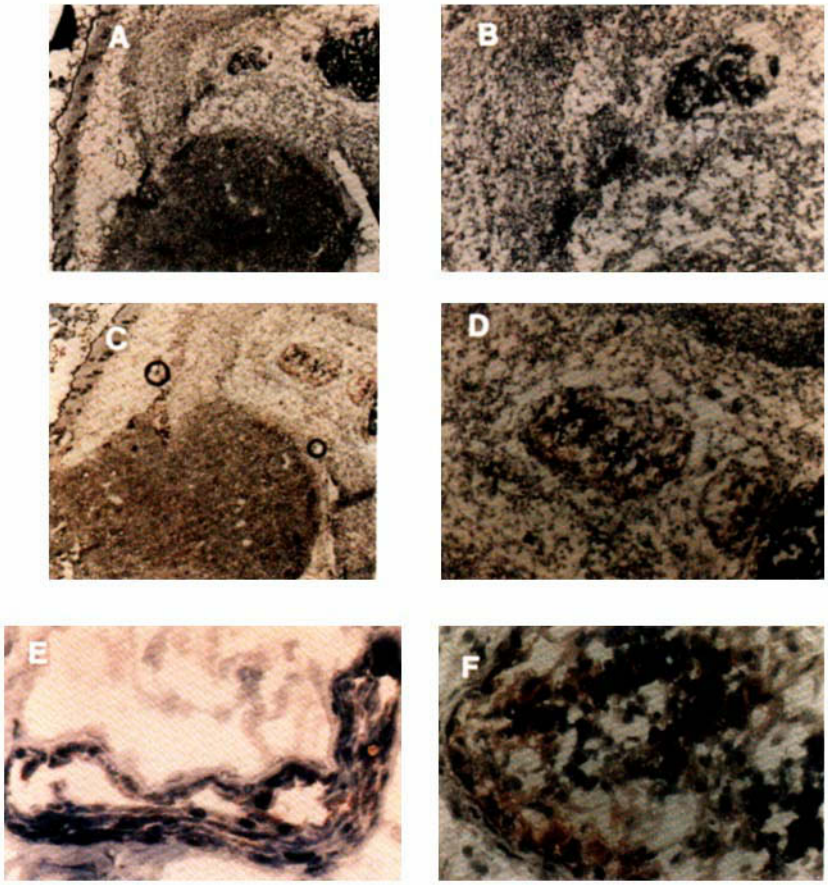

Fig. 5. Localization of Met-72-positive variants within developing B16 subcutaneous melanoma. Biotinylated N-S.7 MoAb bound to a cryostat section of a B16 F1 subcutaneous melanoma (**A**) ×10 and (**B**) ×25. Biotinylated anti-Met-72 MoAb bound to a section of the same B16 F1 subcutaneous melanoma ×10 (**C**), ×25 (**D**), ×100 (**E**), ×128 (**F**).

outgrowth into colonies may be directed by chemotactic or induction factors which are microenvironmentally determined. The fact that both the poorly metastatic B16-F1 and highly metastatic BL6-10 melanoma lines yield a similar distribution pattern after anti-Met-72 staining when grown subcutaneously suggests that considerable re-equilibration of the population occurs during growth as a primary tumor. Details of population re-equilibration and metastatic activity will be the subject of a separate communication (Parratto and Kimura, in preparation).

Our observations in the B16 melanoma model of metastasis are consistent with those of Gabbert et al [24]. They suggest that similar morphologic transitions at the invading front of rat malignant carcinoma may signify a localized process of tumor dedifferentiation. Tumor cell locomotion may be specifically enhanced in regions observed to have a loss of basement membrane and decreased numbers of desmosomes between tumor cells.

An important aspect of these studies focused on isolation and visualization of Met-72-positive variants within metastatic foci of B16 melanoma. The sophisticated

capabilities of the fluorescence-activated cell sorter have provided evidence that Met-72 expression may be a common surface phenotype of B16 melanoma metastatic variants, irrespective of their organ colonization after i.v. inoculation. Experimentally induced ovarian metastases were directly shown to express Met-72, as has been reported for experimental lung metastases of the B16 melanoma [8]. As previously demonstrated with lung-colonizing melanoma cells [25], ovarian colonizing variants were selected by repeated *in vivo* cycling. These variants showed increased, stable levels of Met-72 expression (Fig. 3). Rapid cellular visualization of Met-72 was achieved by two immunocytologic methodologies: 1) cytospin preparations of experimental ovarian metastases, and 2) impression smears of experimental and spontaneous lung metastases. The ease of impression smear immunocytochemistry has permitted rapid characterization of the surface phenotype of cells dislodged from colonized lungs, which are technically refractory to cryostat sectioning. Primary melanomas and other well encapsulated masses do not present suitable specimens for impression smears.

The significant findings of these studies are that 1) Met-72-positive cells are consistently found only at two sites within B16 tumor masses, perivascularly, and at the advancing front and 2) cell surface expression of Met-72 may prove to be a generalized phenotype of B16 melanoma, metastatic variants irrespective of their organ selectivity. The ability to isolate metastatic variant cells from fresh tumor tissue may enable us to quantitate their presence and evaluate acquired qualitative differences during tumor progression and metastatic outgrowth.

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