

Altered Organization of Cell-Substrate Contacts and Membrane-associated Cytoskeleton in Tumor Cell Variants Exhibiting Different Metastatic Capabilities

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ABSTRACT

The pattern of cell-substrate attachment and the organization of actin-containing microfilament bundles were analyzed in tumor cell variants of the K-1735 melanoma and UV-2237 fibrosarcoma series exhibiting distinct metastatic phenotypes. In both tumors, the low-metastatic cells were relatively flat, with well-developed focal contacts. Visualization of the cellular organization of actin and vinculin by fluorescence microscopy indicated that the low-metastatic cells contained prominent stress fibers which terminated in large vinculin-containing focal contacts. The high-metastatic cells were characterized by poor organization of vinculin and adhesion plaques as well as by low number and disarray of actin bundles. These results are compatible with the notion that cytoskeletal organization may be involved in cytodynamic processes related to tumor cell dissemination and implantation.

INTRODUCTION

It is now generally accepted that individual cells within primary neoplasms may be highly heterogeneous with respect to several cellular activities including invasion and metastasis (for reviews, see Refs. 15, 18, and 44). Tumor cell metastasis is defined as the transfer of malignant cells from one organ to another site not directly connected with it. It is still largely unclear what the genetic elements as well as the molecular and cellular factors are that determine and control cell malignancy and expression of metastatic capability.

Results from experimental animal models and analysis of clinical data indicate that many parameters may affect the spread and propagation of metastatic lesions, including release of metastatic cells from the primary tumor, migration into and from blood vessels, seeding in the target organ, and proliferation into new tumor foci. The fate of such cells may also be affected by extrinsic factors such as the host system (15, 18, 44).

Many of the sequential stages in tumor cell metastasis are related to the capability of malignant cells to deform, to form intercellular contacts, and to actively migrate (12, 25, 28, 30, 31, 38-40, 47, 50, 51, 53, 54). These processes are known to be controlled by cytoskeletal networks and, in particular, by the actin-containing microfilament system (42, 56). Thus, it was of interest to determine whether tumor variants which differ in their metastatic potential have altered cytoskeletal organization.

In this report, it is shown that the increased metastatic propensity of 2 recently derived murine tumor cell lines, UV-2237 fibrosarcoma and K-1735 melanoma, is associated with profound reduction in cell-substrate adhesions and with impaired organization of the focal contact-associated proteins vinculin and actin.

MATERIALS AND METHODS

Cell Lines. The UV-2237 is a fibrosarcoma that was induced in a female C3H mouse by chronic UV irradiation (36). The LM³ and the HM cell lines were isolated from the parent UV-2237 tumor (lines cl-15 and ip3, respectively) as described previously (36, 47). The K-1735 melanoma developed in a female C3H⁻ mouse that has been treated with a short course of exposure to UV radiation followed by chronic painting of the skin with croton oil (35). The K-1735-cl-16 (LM) and K-1735-M1 (HM) cell lines were derived from the parent melanoma tumor (16, 17).⁴ These cell variants were kindly provided by Drs. M. L. Kripke and I. J. Fidler, (NCI-Frederick Cancer Research Facility, Frederick, Md.). The distinctive lung implantation capabilities of all the cell lines was reconfirmed by us following i.v. inoculation of 10⁵ cells into syngeneic recipients. The UV-2237-inoculated mice were killed 21 days thereafter, while the mice that were inoculated with K-1735 cells were killed 30 days after injection. Microscope visualization of the lungs revealed that in both cases the HM cells gave a median number of >200 tumor lung nodules per mouse; the median number of LM cells was zero.

Cell Cultures. All the cell lines were grown in monolayers on plastic or glass coverslips in Dulbecco's modified Eagle's minimal essential medium supplemented with glutamine, nonessential amino acids, vitamins, antibiotics, and 10% heat-inactivated fetal bovine serum. The cells were maintained at 37° in a humidified atmosphere of 7% CO₂ and 93% air. To reduce the possibility of appearance of new metastatic variants during cultivation (43), we performed all the experiments with cultures grown no longer than 6 weeks after recovery from frozen stocks. The tumor cells were harvested from cultures in their exponential growth phase by overlaying the cells with a thin layer of 0.25% trypsin-0.02% EDTA for 2 min. Only cell suspensions exhibiting >95% viability (measured by trypan blue exclusion) and free of cell aggregates were used in these studies.

Fluorescent Labeling of Cells. Cultured cells on glass coverslips were briefly extracted for 2 min with 0.5% Triton X-100 in 50 mM morpholinoethane sulfonate buffer, 3 mM EGTA, 5 mM MgCl₂, pH 6.0, and fixed for 30 min with 3% paraformaldehyde (3).

Vinculin was visualized in the cells by double-immunofluorescence microscopy (20) using guinea pig anti-chicken gizzard vinculin and rhodamine-conjugated goat anti-guinea pig IgG. Actin was labeled either with specific antibodies as described (4) or with rhodamine-phalloidin kindly provided by Dr. W. W. Franke (D.K.F.Z., Heidelberg, West Germany).

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³ The abbreviations used are: HM, high metastatic; LH, low metastatic.

⁴ J. E. Talmadge and I. J. Fidler. Enhanced metastatic potential of tumor cells harvested from spontaneous metastases of heterogeneous murine tumor, submitted for publication.

Microscopy. All microscopic observations were performed with a Zeiss photomicroscope III, equipped with filter sets for fluorescein and rhodamine fluorescence and for phase-contrast and interference-reflection optics (3, 4). The experiments were carried out with 2 different batches of frozen cells, and over 100 individual cells were morphologically analyzed prior to the photography of a representative field.

RESULTS

Differences in the morphology and contact patterns were readily observed when K-1735-LM and K-1735-HM variants of the melanoma series were seeded on plastic or on glass (Fig. 1). Phase-contrast microscopy revealed that K-1735-LM cells were well spread on the substrate, with active ruffling membrane at the cell periphery and few or no filopodia (Fig. 1a). The K-1735-HM cells were of irregular shape with many thin filopodia (Fig. 1b, *arrowheads*). The patterns of cell-substrate contact of the 2 cell lines as visualized by interference-reflection microscopy were markedly different. K-1735-LM cells (Fig. 1c) were associated with the solid substrate via many large and well-developed focal contacts⁵ and peripheral dots (both of which exhibit interference-reflection dark images). Cells of the K-1735-HM had apparently a smaller number of defined focal contacts, and the ones detected were usually of smaller size (Fig. 1d). These cells, however, often formed close contacts (interference-reflection gray) with the substrate, which are most prominent in the elongated cytoplasmic processes and filopodia.

Actin organization was visualized by staining with either specific antibodies or with rhodamine-labeled phalloidin. The actin system of K-1735-LM cells is organized in tightly packed filament bundles near the ventral cell membrane (Fig. 2a). These microfilaments apparently terminated in cell-to-substrate focal contacts, as may be appreciated from the fluorescence picture (Fig. 2a) and the corresponding interference-reflection image (Fig. 2b). A markedly different organization was seen in the K-1735-HM cells; bundles were hardly detected near the ventral cell membranes (Fig. 2c), and few residual filamentous arrays were found in a higher focal plane above the nucleus (Fig. 2d). Interference-reflection images of these permeabilized and fixed HM cells were almost completely abolished (Fig. 2e) [compare the interference reflection photomicrographs of viable K-1735-HM cells (Fig. 1d)]; the loss of interference reflection upon permeabilization is a general phenomenon found in all cell types. Vinculin visualized in K-1735-LM and -HM cells (Fig. 2, *f* and *h*, respectively) show overlapping patterns with the interference reflection images (Fig. 2, *g* and *i*). It should be noted that the cells labeled for vinculin were obviously permeabilized and thus that some of the small focal contacts lost their contrast; this seems to be the reason for the only partial coincidence between the 2 images, vinculin and interference reflection. The close relationships between intracellular, membrane-bound vinculin and cell contacts (both cell-substrate focal contacts and related cell-to-cell junctions) as well as its involvement in the linkage of actin bundles to the membrane were demonstrated previously in a variety of cells and tissues (20–24).

⁵ The development of interference-reflection microscopy, either alone or in conjunction with immune fluorescence, pointed to 2 general types of contacts. In interference-reflection dark areas defined as focal contacts, the cell membrane is separated from the substrate by a gap of 100 to 150 Å while interference-reflection gray areas represent close contacts in which the membrane is ~300 Å away from the substrate (1, 20, 32–34).

The shapes and contact patterns of the UV-2237 fibrosarcoma cell variants were generally different from those of the K-1735 melanoma cells. Nevertheless, the differences between the cell-substrate contacts and cytoskeletal organization observed in the UV-2237-LM and -HM cell variants followed a similar pattern to that detected in the K-1735 melanoma system. The UV-2237-LM cells were relatively flat with many prominent focal contacts (Fig. 3, *a* and *c*, respectively), while the cells of the HM line were more elongated and cylindrical with reduced number of focal contacts (Fig. 3, *b* and *d*, respectively). This observation was further corroborated by actin and vinculin labeling as shown in Fig. 4. Staining of the UV-2237-LM cells for actin using either actin antibodies or rhodamine-phalloidin revealed the existence of elaborate networks of elongated stress fibers (Fig. 4a) which apparently terminated in vinculin-rich focal contacts (Fig. 4). The actin of the HM variant cells was poorly organized, exhibiting only a few distorted arrays of microfilaments (Fig. 4c). Vinculin in UV-2237-HM cells was organized mostly in spots and patches which were considerably smaller than the typical focal contacts of the UV-2237-LM cells. It should be pointed out that within the UV-2237-HM cell population we occasionally detected a few cells or cell groups which exhibited well-developed actin stress fibers. These cell variants were usually in the order of 5% and could represent the heterogeneity within the UV-2237-HM cell line (ip3), which was selected from the parent tumor by 3 *in vivo-in vitro* cycles without further cloning (48).

DISCUSSION

The process by which malignant cells disseminate from the primary tumor to form distant metastases is probably among the most critical phenomena in clinical oncology. The metastatic process consists of many steps including detachment of cells from the primary tumor mass, invasion into the circulation, movement through the capillary bed, extravasation into the target organ, proliferation in that site and the interaction, throughout the entire process, with the host immune system (15, 18, 44). This complexity renders it unlikely that the development of metastatic foci will depend upon one ubiquitous factor. Nevertheless, during the last decade, many experimental attempts were made to correlate different properties of malignant cells with their metastatic capabilities. Among those are the production of degradative enzymes and their secretion by the cells (11, 37, 57), expression of cell surface components (7, 10, 19, 27, 39, 45, 47, 49, 51, 52), adhesion and intercellular recognition (5, 31, 40, 47, 59), metabolic response to cell shape alteration (5, 46) and resistance to cytotoxic cells of the host (26, 29, 55). In light of the nature of the metastatic process whereby tumor cells move from one organ to another through the circulation, it is conceivable that dynamic properties and mechanochemical deformability of tumor cells may play a central role in the metastatic process. This notion, together with the fact that treatment with cytoskeleton-disrupting drugs induce marked alterations in the metastatic properties of tumor cells (26, 30, 38), motivated us to compare the patterns of cell-substrate adhesion and cytoskeletal organization in cultures of established tumor cell variants exhibiting HM or LM capabilities.

Previous analysis of cells derived from human malignant lesions failed to reveal any conclusive evidence regarding the

organization or the cytoskeleton in those cells, probably due to their heterogeneous cell populations (6). We have performed the experiments described above using 2 recently derived, well-established murine tumor cell systems in order to gain further knowledge on the possible involvement of the cytoskeleton in tumor cell metastasis.

To probe the 3 major cytoskeletal networks, namely, microfilaments, microtubules, and intermediate filaments, we have used antibodies to actin or rhodamine-conjugated phalloidin, tubulin antibodies, and vimentin antibodies, respectively. Among these systems, significant differences between HM and LM variants were detected predominantly in the microfilament network of both tumor systems tested. The HM cell lines exhibited distorted actin bundles accompanied by marked reduction in the number and organization of vinculin-rich focal contacts. It has been demonstrated previously that vinculin is specifically associated with cell substrates, focal contacts, and related structures *in vivo*, where it is probably involved in the linkage of actin filament bundles to the plasma membrane (14, 20-24). Differences existing between the microtubular and intermediate-filament systems of the HM and LM cell lines of both tumors were usually minor and are believed to be secondary to shape variations of the different cells (not shown).

The variability in actin organization within each line was quite limited; nearly all the LM cells exhibited prominent actin bundles while nearly all the cells in the HM variants had poor actin organization at the ventral region of the cell and only few distorted filaments at the dorsal side.

It is noteworthy that many studies performed over the last years associated the transformation of cells by oncogenic viruses with dramatic alterations of the microfilament system (2, 9, 13, 41, 58). The observed changes in actin organization in such transformed cells were similar to those described herein for the HM cells, namely, the HM tumor cell variants (both K-1735 melanoma and UV-2237 fibrosarcoma) exhibit morphology, contact pattern, and microfilament organization which are reminiscent of those described for virally transformed cells while the LM variants are more compatible with the "nontransformed phenotype." However, unlike the situation in those studies where "normal cells" are compared with their "transformed counterparts," all the cell lines tested here fulfill the *in vitro* and *in vivo* criteria of transformed cells inasmuch as they are not contact inhibited, grow in semisolid medium, and are tumorigenic.

Thus, it is possible that the loss of stable cellular contacts and the disarray of actin bundles often attributed to transformed cells are not strictly related to the transformation *per se* and may reflect the malignant metastatic potential of these cells.

At present, we still do not know how the variations in cell contacts and actin organization which we have detected in cultured cells are expressed in the different tumor cell lines growing in 3 dimensions *in vivo* and by what mechanisms the alterations in cytoskeletal organization contribute to the expression of the metastatic phenotypes. It is nevertheless likely that a decrease in cell contacts and in actin organization will enable the cells to distort their shape during their migration through and across small capillaries. Similarly, studies with normal cells, like migrating fibroblasts and macrophages, which are capable of rapid migration both *in vivo* and *in vitro*, indicate lack of defined stress fibers which are more common in more

stationary cells such as fibroblasts and epithelial cells (for discussion, see Ref. 8).

Although the differences reported here for the cytoskeleton organization in HM and LM cell lines of 2 different parental tumors are prominent, the generality of these findings is yet to be determined. If, indeed, it will be established that the organization state of the microfilament system is indicative of the cell metastasis potential, then such an approach may provide a better understanding of the process of tumor progression and may prove to be of prognostic value in predicting the metastatic potential of cell subpopulations within individual tumors.

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Fig. 1. Phase-contrast (a and b) and interference-reflection (c and d) photomicrographs of melanoma K-1735-LM cells (a and c) and the K-1735-HM cells (b and d). Arrowheads, filopodia on K-1735-HM cells (b); arrows, typical elongated focal contacts in K-1735-LM (c). \times 950.

Fig. 2. Distribution of actin (a, c, and d) and vinculin (f and h) in melanoma cell lines K-1735-LM (a and f) and K1735-HM (c, d, and h). The cells were permeabilized with detergent, fixed, and then fluorescently labeled. Actin was visualized here with rhodamine-phalloidin (1.5 μ g/ml) and vinculin with guinea pig antibodies to vinculin (30 μ g/ml) and rhodamine-goat anti-rabbit immunoglobulin (10 μ g/ml). In each case, the same field was photographed with interference-reflection optics (b, e, g, and i). The cells shown in c and d were photographed in 2 focal planes, dorsal focal plane above the nucleus (c) and ventral focal plane close to the substrates attached membrane (d). Notice the overlap between the vinculin patches and focal contacts (matched arrowheads in f and g) and the loss of contact images after the fixation in the K-1735 cells HM (e and i). \times 950.

Fig. 3. Phase-contrast (a and b) and interference-reflection (c and d) photomicrographs of fibrosarcoma UV-2237-LM (a and c) and UV-2237-HM cell variants (b and d). The LM cells grow in well-spread fashion with prominent focal contacts (arrows in c) while the HM variant is loosely attached and exhibits only sparse attachment plaques (arrows in d). In b and d, the same field was photographed. \times 950.

Fig. 4. Distribution of actin (a and b) and vinculin (c and d) in the UV-2237-LM (a and c) and UV-2237-HM fibrosarcoma (b and d) variants. Actin and vinculin were labeled as detailed in the legend to Fig. 2. \times 950.







