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Motility and Adhesive Properties of High- and Low-Metastatic Murine Neoplastic Cells

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ABSTRACT

Randomly chosen clones of the murine K-1735 melanoma tumor were used by Raz and Geiger to address the question of whether variations in actin organization in these cells may be related to their lung colonization capability in syngeneic hosts (Cancer Res., *42:* 5183–5190, 1982). In 14 of 15 clones tested, we found that the degree of actin organization was inversely correlated to their metastatic capability. We have further shown that remarkable variations exist in the adhesive properties and locomotor activity of four K-1735 melanoma cell variants that exhibit distinct metastatic properties. The low-metastatic cell variants displayed large focal adhesion plaques, tightly packed actin bundles, elaborate extracellular networks of fibronectin, and restricted motility. In contrast, the high-metastatic variants were poorly attached with only few distinct actin bundles, were unable to reorganize extracellular fibronectin into cables, and exhibited high motile activity. Electron microscopic examination of local s.c. tumors of the high- and low-metastatic lines indicated that the former formed loose tumor masses with very few intercellular connections, while the low-metastatic line developed into a considerably more compact tumor with numerous intercellular contacts, in line with the *in vitro* findings.

It is proposed that the differences in cellular properties manifested by these cell lines may be related to their metastatic properties. Specifically, the highly metastatic cells of this tumor system may easily detach from the primary tumor mass, form weak and transient connections with surrounding connective tissue, and actively migrate through it. Furthermore, these results point to the close interrelationships between different mechanochemical features in cells, including specific cell adhesiveness, cytoskeletal organization, locomotion, and rearrangement of extracellular fibronectin. The possible nature of these interrelationships is discussed.

INTRODUCTION

Metastasis, the dissemination of malignant diseases, involves linked, sequential steps which are determined by both unique properties of the metastatic progenitor cells and their interactions with the host (11, 24, 29). The detachment of tumor cells from the primary site of growth is a necessary preliminary step for metastasis. This release depends on the capability to break existing contacts with neighboring cells or connective tissue. The tumor cells may then invade the surrounding tissues and penetrate as either single cells or small cell clumps into blood vessels or the lymphatic system, where they may be transported to distant sites. The arrest of these cells in organs may be influenced by the formation of tumor emboli as a result of self-aggregation and/or interaction with circulating host cells (14, 22, 30, 31, 34). The site of arrest and subsequent proliferation of the tumor cells may be either specific or nonspecific; the notion of site-specific metastatic spread stems from observations of a nonrandom distribution pattern of metastases (27, 28, 45) and from the presence of tumor cell variants exhibiting preferential colonization in specific organs (7, 13, 33, 36, 41). It was assumed that specific tumor cell arrest depends on the interactions of tumor cells with capillary endothelium or with exposed basement membranes and intercellular matrix (20, 25, 43). The attainment of an extravascular position is believed to involve an active locomotion similar to that responsible for the initial invasion into the blood vessels.

In light of the involvement of cell adhesiveness and motility throughout the metastatic spread, we have examined recently several pairs of tumor cell variants exhibiting either low- or high-metastatic potential (32). We have found that the low-metastatic cell lines of the 2 types of tumors form extensive substrate contacts, well-developed vinculin-containing adhesion plaques, and tightly packed networks of actin bundles. The corresponding high-metastatic cell lines were relatively loosely attached, with fewer and smaller vinculin-containing focal contacts and poorly organized actin filaments (32). Our initial observations raised 2 major questions. (*a*) Are the apparent variations in the adhesive properties and cytoskeleton primary manifestations of the "metastatic phenotype" or do they reflect possible preexistng differences, such as variations in adhesion-promoting activities of extracellular matrix components produced by these cells? (*b*) Do the high- and low-metastatic variants differ in their cytodynamic activity?

In this paper, we show by direct examination of cell locomotion *in vitro* that the high-metastatic cell variants of the K-1735 melanoma exhibit much higher locomotor activity that do their low-metastatic counterparts. We show that both high- and low-metastatic cell variants of this tumor system produce fibronectin and interact with it. However, while the low-metastatic variants organize it into cables, this matrix protein remains unperturbed and uniformly distributed in cultures of the high-metastatic variants. Plating of cells on solid substratum coated with fibronectin does not alter the intracellular distribution of either actin or vinculin as compared to cells growing on uncoated substrate, suggesting that these differences in the primary adhesive properties of the cells are not induced by extracellular matrix. In addition, examination of cells in s.c. tumors of the high- and low-metastatic lines at the electron microscope level indicated that the former were considerably more compact. The causal interrelationships between the different cellular manifestations of the metastatic phenotype and their possible effect on the process of metastatic spread are discussed.

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MATERIALS AND METHODS

Cell Culture. All the cell lines were grown in monolayer cultures on plastic dishes or on glass coverslips in CMEM.[3] The cells were maintained at 37° in a humidified atmosphere of 7% CO_2 and 93% air. To ensure reproducibility, all experiments were done with cultures grown for no longer than 4 weeks after recovery from frozen stocks. The tumor cells were harvested from cultures in their exponential growth phase by overlaying the cells with 2 mM EDTA in PBS free of Ca^{2+} and Mg^{2+}. Cell viability was measured by trypan blue exclusion, and only cell suspensions exhibiting >95% viability and freedom from cell aggregates were used in these studies.

Tumor Cell Variants. The K1735 Cl-11 and K-1735 Cl-16 (low incidence of lung colonization after i.v. or s.c. inoculation) lines were obtained by cloning of the fifth *in vitro* passage of the parental K-1735 melanoma. The K-1735-M1 and K-1735-M4 (high incidence of lung colonization) lines were both derived independently from pulmonary metastases after i.v. inoculation of the parent tumor cells (12, 40).

Colonial Growth of Cells in Semisolid Medium. Cell suspensions of K-1735 melanoma were seeded at a density of 10^3 cells/60-mm dish in 0.5% agarose (Seaplaque FMC Corporation, Marine Colloids Division, Rockland, Maine) in CMEM on top of a 1% agarose layer. The dishes were then incubated at 37°, and 18 days later, individual colonies were transferred into culture wells (Costar 3542 plates). Following 3 *in vitro* passages, the cell monolayers were harvested, and samples were either plated on coverslips and labeled for actin or injected i.v. (10^5 cells) into syngeneic recipient mice. Thirty days after the injection, the number and size of lung metastases were determined macroscopically and microscopically.

Fibronectin. Human plasma fibronectin (CIG) was isolated by affinity chromatography on Sepharose-bound gelatin according to the procedure of Ruoslahti *et al.* (35) as described earlier (6). Fibronectin carpets were prepared by incubating tissue culture dishes or glass coverslips with pure CIG (15 μg/ml in PBS) for 60 min at 24°C. The CIG-coated substrates were rinsed extensively with PBS before use (6).

Assays of Cell Attachment and Spreading. Tumor cells were suspended at a concentration of 3×10^6 cells/ml in serum-free CMEM, containing 1% BSA, and radiolabeled for 2 hr at 37° with 5 μCi of carrier-free $Na^{51}CrO_4$ (New England Nuclear). At the end of the incubation, the cell suspensions were washed extensively and plated in quadruplicates in 16-mm Costar culture dishes, either uncoated or coated with human CIG. After various time intervals, the cultures were washed gently with PBS, and the attached cells were solubilized with 0.1 N NaOH (30 min, 24°). The cell-associated radioactivity was determined in a Packard Auto-Gamma scintillation spectrometer.

Immunochemical Reagents and Procedures. Antibodies to vinculin were prepared in guinea pigs by repeated injections of pure chicken gizzard vinculin and purified on vinculin immunoadsorbent as described (15, 17). Antibodies to CIG were raised in rabbits and affinity purified (6). For the visualization of actin, we used here rhodamine-conjugated phalloidin which specifically binds to F-actin (kindly provided by Dr. H. Faulstich, Max-Plank Institute, Heidelberg, Germany). For the staining of extracellular fibronectin, the cultures were fixed with 3% paraformaldehyde in PBS or permeabilized first with detergent {0.5% Triton X-100 in 50 mM *N*-morpholinoethanesulfonate buffer-5 mM $MgCl_2$-3 mM [ethylenebis(oxyethenenitrilo)]tetraacetic acid, pH 6.0} and then fixed. Staining for actin and vinculin was carried out after similar permeabilization and fixation.

Phagokinetic Tracks. Uniform carpets of gold particles were prepared on glass coverslips coated with BSA or with CIG essentially as described by Albrecht-Buehler (1, 2) (for the fibronectin-coating procedure, we omitted the ethanol treatment). The coverslips were rinsed extensively to remove nonadhering or loose gold particles before cell plating. Phagokinetic tracks were visualized using low-power objectives (×4 to ×16) and a dark-field condenser (0.7 to 0.85) in a Zeiss Photomicroscope III. To compare fluorescent patterns to the distribution of gold particles, we used a high-power objective (Zeiss Planapochromat; ×63/1.4). Fluorescence was visualized using the epiilluminator as described (6), and the gold particles were detected as dark dots using bright-field optics. The 2 images could easily be correlated when the specimen was exposed to the 2 light sources simultaneously.

Electron Microscopy. Solid s.c. tumor masses were cut into 1- to 2-mm blocks, fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and postfixed with 1% OsO_4 in the same buffer, then dehydrated, embedded in Epon, and sectioned. The sections were stained with uranyl acetate and lead citrate according to standard procedures and examined in a Phillips EM 300 microscope at 80 kV.

RESULTS

Relationships between Cytoskeletal Organization and Metastatic Capability of Cloned K-1735 Melanoma Cells. We have reported previously (32) that established metastatic variants of K-1735 melanoma differ from their low-metastatic counterparts in their cytoskeletal organization. This was manifested by low number and disarray of actin bundles in the former. This observation raised the question as to whether, in this particular tumor system, the degree and pattern of actin organization may be a reliable indicator for the prediction of the metastatic potential of individual cells or cell clones.

Fluorescence staining of cell monolayers of randomly selected clones revealed that the cells could be divided into 3 major groups in which actin was either well organized (Fig. 1*A*), shows intermediate level of organization (Fig. 1*C*), or was poorly organized (Fig. 1*E*). The different clones were then harvested, and 10^5 cells were injected via the tail vein into syngeneic recepients.

A comparison between the results obtained from the labeling of the various clones for actin and the independent determinations of their respective metastatic activity after i.v. inoculation is depicted in Fig. 1, *B*, *D*, and *F*, and summarized in Table 1. As shown, a correlation could be found between the degree of actin organization and the number of pulmonary metastases. Thus, all the clones which exhibited poor organization of actin (see Fig. 1, *C* and *E*) gave relatively high metastatic load in the animals

Table 1

Correlation between organization of actin in cloned cultured melanoma cells and their metastatic potential

Each mouse was inoculated i.v. with 10^5 cells.

Source of cells	Organization of actin	Pulmonary metastases[a]		
		Median	Range	Size
Clone 38	Poorly organized	>200	6–>200	Variable
Clone 42	Poorly organized	>200	>200	Variable
Clone 66	Poorly organized	>200	>200	Small
Clone 72	Poorly organized	33	10–>200	Variable
Clone 78	Poorly organized	20	7–>200	Large
Clone 14	Intermediate	28	16–>200	Variable
Clone 25	Intermediate	18	6–36	Medium
Clone 31	Intermediate	16	6–27	Variable
Clone 46	Intermediate	57	16–>200	Variable
Clone 77	Intermediate	24	3–43	Large
Clone 3	Well organized	3	0–16	Medium
Clone 17	Well organized	5	0–11	Large
Clone 21	Well organized	5	0–14	Variable
Clone G	Well organized	>200	27–>200	Large
Clone D	Well organized	6	0–30	Small

[a] Ten recipient mice were used for each group.

[3] The abbreviations used are: CMEM, Dulbecco's modified Eagle's medium supplemented with glutamine, nonessential amino acids, vitamins, antibiotics, and 10% heat-inactivated fetal bovine serum; PBS, phosphate-buffered saline [NaCl (80 g/liter)-KCl (0.2 g/liter)-$NaHPO_4$ (1.5 g/liter)-KH_2PO_4 (0.2 g/liter)-$MgCl_2$ (0.2 g/liter)-$CaCl_2$ (0.1 g/liter) (pH 7.2)]; CIG, human plasma fibronectin; BSA, bovine serum albumin.

receiving injection, while those clones in which actin was organized in large cables, with the exception of clone G, gave no or only few metastases. It should be emphasized that the determination of cytoskeletal organization was performed on several samples from each tumor clone and was totally independent of the *in vivo* assay of experimental metastases formation. On the basis of these results, it was concluded that, in this particular tumor system, variations in the organization of the cytoskeleton are of major importance in the determination of the intrinsic metastatic potential of individual cells.

Locomotor Activity of High- and Low-Metastatic Cell Variants. Two low-metastatic (Nos. 11 and 16) and 2 high-metastatic cell lines (M1 and M4) of the K-1735 melanoma tumor were plated on gold particle-coated substrates. After different time intervals, the cells were fixed and examined in dark-field optics. Comparison of the phagokinetic tracks produced by the 4 cell lines indicated that the low-metastatic variants were essentially stationary. Gold particles were collected rapidly from their immediate vicinity, with only slight enlargement of the diameter of the cleared zone, upon prolonged incubation. The high-metastatic lines M1 and M4, on the other hand, exhibited considerable locomotor activity, as apparent from the phagokinetic tracks produced by the cells after 1 or 4 days of culture. Pictorial demonstration of these differences is shown in Fig. 2.

To obtain some idea as to the quantitative differences in mobility, we plated cells on gold-coated substrates and measured the areas cleared by cultured cells of the 2 types of lines. This was done by monitoring the microscopic image on a television screen and tracing the images on a semitransparent paper of uniform thickness. The papers (as well as paper squares representing known areas) were weighed, and the average areas of the tracks were calculated.

The average area of the particle-clear zone in the low-metastatic Cl-11 cells (calculated from 50 unselected cells) after 24 hr of incubation was 2900 ± 1450 sq μm, while the area of the tracks formed by the high-metastatic M1 cells was 7400 ± 3300 sq μm.

Examination of the phagokinetic tracks at a higher magnification indicated that the low-metastatic lines removed the gold particles from the substrate much more extensively than did the high-metastatic ones (not shown). Many gold particles could be detected along the migratory tracks of M1 and M4 cells, while the clearance of particles from the immediate vicinity by clones 11 and 16 was almost complete.

To test whether the correlation between high locomotor activity *in vitro* and high-metastatic potential is a general phenomenon present in other tumor systems too, we have examined 8 independent cell lines derived from 2 additional tumor systems (UV-2237 fibrosarcoma and B16 melanoma). The results, however, indicated that there was no predictable correlation between low-metastatic potential and restricted mobility in these 2 tumor systems (not shown, see "Discussion").

Organization of Fibronectin in K-1735 Cell Variants. In light of the involvement of the extracellular matrix protein fibronectin in cell adhesion and locomotion (19, 46), we have examined its distribution in metastatic variants of K-1735 melanoma. Cultures of the different variants of this tumor were permeabilized, fixed, and labeled immunofluorescently for fibronectin. The results depicted in Fig. 3 show that cultures of the 2 low-metastatic clones 11 and 16 contained many conspicuous fibronectin cables, which could be visualized already 24 hr after plating (Fig. 3*A*), and became most prominent within a few days thereafter (Fig. 3, *C* and *E*, respectively). The high-metastatic cell lines of the same parental tumor showed only diffuse fibronectin labeling, and even 5 days after plating, only a few short fibronectin filaments were detected, as shown in Fig. 3, *B*, *D*, and *F*.

These observations raised the question of whether the distinct pattern of fibronectin in high- and low-metastatic variants reflect quantitative differences in the production of fibronectin or a different mode of rearrangement. Cultures of the 4 lines of K-1735 melanoma were incubated for 16 hr in CMEM containing [^{35}S]methionine (40 μCi/ml). The medium was then supplemented with 50 mM methionine and dialyzed exhaustively against PBS. The cells were extracted with small volumes of 8 M urea and 5 mM dithiothreitol (0.2 ml/3.5-cm culture dish), and the extract was further diluted 20-fold in PBS. Samples of the cell extracts (corresponding to the same numbers of cells) and of the dialyzed medium were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiograms of the cell-associated, electrophoretically separated polypeptide were indistinguishable (Fig. 4, *cells*), including peptides comigrating with CIG (*arrowheads*). Analysis of the medium (Fig. 4, *sup*) indicated that the major polypeptides released had an apparent molecular weight of 220,000 and comigrated with purified fibronectin. The M_r 220,000 bands on the sodium dodecyl sulfate gels were identified as fibronectin by immunoprecipitation with fibronectin-specific monoclonal antibody, by immunoblotting analysis, and by specific absorption on Sepharose gelatin column (not shown).

Densitometric scanning of the autoradiograms as well as determination of the relative quantities of radioactively labeled proteins which could be adsorbed by Sepharose-bound gelatin (obtained in 3 different determinations) indicated that the amounts of fibronectin released by the low-metastatic cells to the mediums were nearly double, as compared to the high-metastatic cells.

Could these quantitative differences in fibronectin production and release be the primary cause for the observed variations in cell morphology and mobility? To determine the effect of exogenous fibronectin on these properties, we have seeded all 4 cell lines on CIG-coated substrates. The distribution of actin in clone 11 (low metastatic; Fig. 5, *A* and *C*) and in M1 cells (high metastatic; Fig. 5, *B* and *D*) is shown, plated on fibronectin carpets (Fig. 5, *A* and *B*) or on regular uncoated coverslips (Fig. 5, *C* and *D*). It was found that coating of substrate with fibronectin did not lead to an alteration in actin organization in these cells. Specifically, the addition of fibronectin to the high-metastatic cells did not change their general morphology, their pattern of substrate contacts, and cytoskeletal organization. Moreover, plating of high-metastatic cells on extracellular matrix produced by either primary human foreskin fibroblasts or K-1735 Cl-16 did not alter cytoarchitecture to a significant extent. It will be shown below that the presence of large amounts of fibronectin on the substrate had no apparent effect also on the locomotor activity of these cells.

Several explanations could be offered to account for the inability of substrate-attached fibronectin to "induce" low-metastatic morphology and behavior in cultured cells. It could be argued, for example, that the high-metastatic cells are unresponsive to the adhesion- and spreading-promoting activity of fibronectin due to possible absence of fibronectin-specific receptors

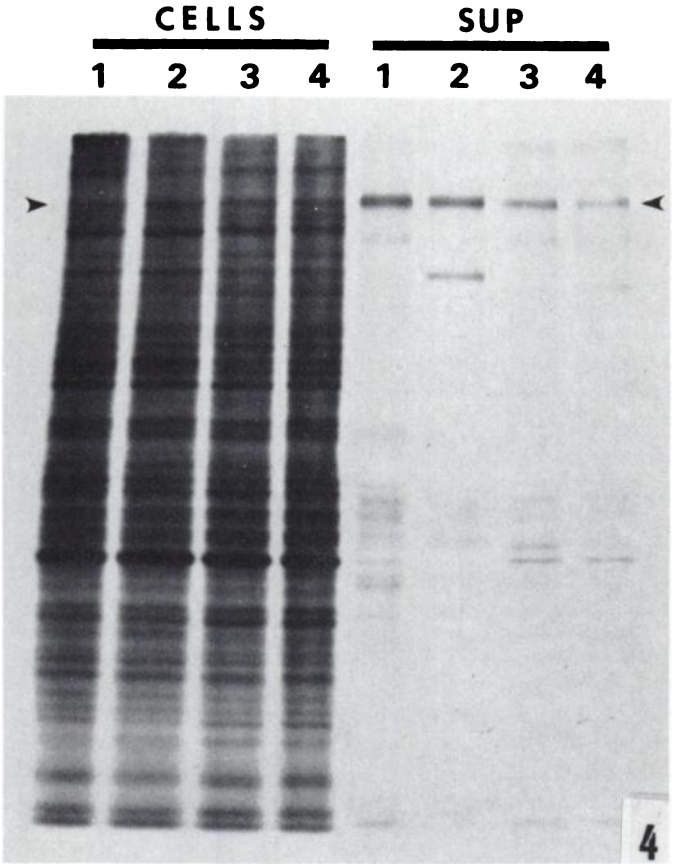

Fig. 4. Autoradiogram of electrophoretically separated, biosynthetically labeled ([^{35}S]methionine) polypeptides associated with cells (*CELLS, Lanes 1* to *4*) or released to the culture medium (*SUP, Lanes 1* to *4*). *Cells: 1*, clone 11; *2*, clone 16; *3*, M1; *4*, M4 incubated for 16 hr with 40 μCi [^{35}S]methionine per ml. The medium was then supplemented with 50 mM unlabeled methionine, collected, and dialyzed against PBS, and the cells were rinsed and lysed in electrophoresis sample buffer. After removal of free methionine, electrophoresis sample buffer was added to the supernatant. Aliquots corresponding to the same numbers of cells were analyzed by sodium dodecyl sulfate electrophoresis on a gradient polyacrylamide gel (6 to 15%). The pattern of cell-associated polypeptides was apparently indistinguishable in all 4 lines. In the medium, the major polypeptide released was fibronectin. *Arrowheads*, location of a human plasma CIG used as a marker. The amounts of ^{35}S-labeled fibronectin released by the low-metastatic lines (Nos. 1 and 2) were nearly double those of the high-metastatic lines (Nos. 3 and 4), as determined by densitometric tracing of the autoradiogram.

on their surface. To address this aspect, we have plated the 4 cell lines (in serum-free medium supplemented with BSA) onto fibronectin-coated or uncoated coverslips and determined the rate of their substrate attachment and extent of spreading. Chart 1, *solid lines*, shows the adhesion profile of ^{51}Cr-labeled K-1735 variants on a fibronectin carpet. It is apparent that all 4 variants (both high and low metastatic) adhered rapidly to the fibronectin-coated surface. In the absence of fibronectin (Chart 1, *broken lines*) or in the presence of irrelevant proteins such as BSA, only a limited degree of cell attachment could be detected. These results suggested strongly that all the melanoma variants bind specifically to fibronectin; in other words, they all contain some sort of fibronectin receptors. Moreover, microscopic examination of the substrate-adherent cells from such experiments showed extensive spreading on the fibronectin carpet.

Active Rearrangement of Substrate-attached Fibronectin by Cultured Metastatic Variants. In an attempt to explore the basis for the different patterns of fibronectin displayed by the low- and high-metastatic variants of the K-1735 melanoma, we have plated the cells on a uniform carpet of CIG, and after different time intervals, we have permeabilized and immunolabeled them for fibronectin. The results (Fig. 6) indicated that the low-metastatic cells (represented here by clone 11) progressively removed the underlying fibronectin from the substrate and reorganized it into cables (Fig. 6*A*), mostly under and between the cells. The high-metastatic M1 cells did not perturb the fibronectin carpet, nor did they exhibit organized fibronectin filaments as shown in Fig. 6, *B* and *D*. The pattern of fibronectin in corresponding cultures seeded on uncoated substrates is depicted in Fig. 6, *C* and *D*.

To further characterize the relationships between adhesiveness and mobility in the 2 types of metastatic variants, the cells were plated on fibronectin-coated glass coverslips, which were further coated with gold particles. We show representative examples of the low-metastatic K-1735 Cl-11 (Fig. 7, *A*, *C*, and *E*) and the high-metastatic K-1735 M1 cells (Fig. 7, *B*, *D*, and *F*). For each clone, the same cell was photographed using either dark-field optics with a low-power (×16) objective (Fig. 7, *A* and *B*), immunofluorescence with a high-power (×63) objective (Fig. 7, *C* and *D*), or high-power (×63 objective) combination (Fig.7, *E* and *F*) of bright-field and fluorescence optics to match the 2 images (see "Materials and Methods"). The differences in motility of the 2 cell types, as depicted in Fig. 2 above, were not altered significantly when fibronectin was used as an anchoring substrate for the gold particles instead of BSA as pointed out above. Namely, the low-metastatic counterparts were motile. The removal of gold particles by the former from their immediate vicinity was extensive, while the M1 cells removed it only partly. Moreover, clone 11 cells removed the underlying fibronectin, while the M1 cells hardly perturbed it. The comparison of the gold particle-free area with the area from which fibronectin was removed (Fig. 7*E*, *broken line*) indicated that the gold particles were displaced from larger areas than fibronectin, suggesting that the 2 were manipulated independently. One can thus distinguish between superficial contacts which are sufficient to remove the gold particles and the formation of focal contacts which is accompanied by reorganization of extracellular matrix proteins.

Patterns of Intercellular Contacts in the Intact Tumor Tissue *in Vivo*. The properties of the various metastatic variants de-

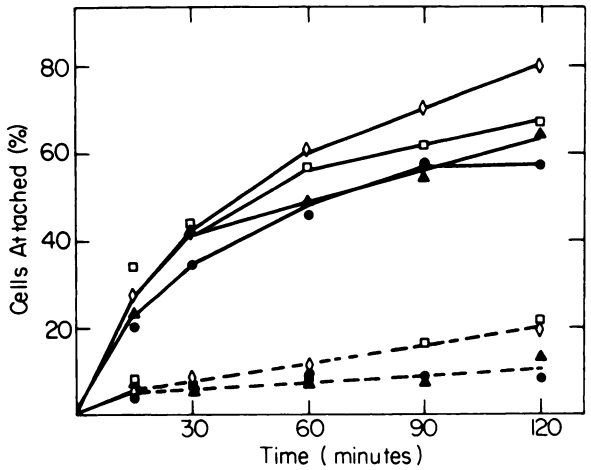

Chart 1. Time course for the attachment of K-1735 melanoma cell variants to plastic (- - - -) and CIG (——)-coated dishes. ◇, K-1735 M1; □, K-1735 M4; ▲, K-1735 Cl-11; ●, K-1735 Cl-16.

scribed above were mostly based on *in vitro* experiments carried out with cultured cells. To examine whether the relatively reduced adhesivity observed in culture is also typical for tumors produced by the high-metastatic lines, local tumors of the 2 types of lines of K-1735 melanoma (high and low metastatic) were examined at the electron microsope level. The results depicted in Fig. 8 show that the 2 were distinctly different. The low-metastatic line Cl11 formed compact tumor with little connective tissue between the cells and a large number of tight intercellular connections (Fig. 8*A*). High-power magnification revealed that, in many of these contact areas, submembranal density, similar to that found in typical adherens-type junctions, could be detected (Fig. 8*B*). The high-metastatic line M1 formed relatively loose tumors with a large proportion of intercellular connective tissue (Fig.8*C*) with only few contacts between the cells (Fig. 8*D*).

DISCUSSION

Many studies over the last several years were aimed at the identification and characterization of metastatic processes at the molecular and cellular levels (for reviews, see Refs. 11, 24, and 29). Such studies, and especially those in which high- and low-metastatic variants of the same tumors were used, suggested that a large number of factors may be involved in the spread of neoplastic cells throughout the body, some of which are extrinsic such as the immune system of the host, vascularization, etc., while others are intrinsic and depend predominantly on the activity of the individual tumor cell. It seems most probable that among these intrinsic factors which may affect the metastatic potential is the capacity of cells to break existing contacts and to actively move (9, 18, 39).

This rationale was supported by results obtained in our previous study on the cytoskeletal organization found in metastatic variants derived from the same parental tumors (32). We have shown that cells of UV-2237 fibrosarcoma and K-1735 melanoma exhibiting low-metastatic properties had large focal contacts, associated with vinculin and with conspicuous actin bundles, in contrast to their high-metastatic counterparts. To further substantiate these findings, we have taken in the present study the reverse approach. We have first determined the degree of actin organization in randomly selected K-1735 cells and then determined independently their respective lung colonization capability after i.v. inoculation. We have found that, in 14 of 15 clones studied in detail, there was a correlation between patterns of actin distribution and metastatic potential. This suggested that, at least in this tumor system, the organization of actin *in vitro* reflects the expected metastatic activity *in vivo*. More specifically, it indicated that, among the large variety of intrinsic and exogenous factors which may affect metastatic dissemination in general, the structures of the cytoskeleton and the related dynamic properties of the cells play a major role in defining the metastatic activity of K-1735 melanoma cells. It was thus of interest to further explore the dynamic properties of these cells. The present results indicate that, while the low-metastatic clones 11 and 16 in culture were essentially stationary, the high-metastatic variants showed considerable locomotor activity.

Among the other differences noted here was the rearrangement of extracellular fibronectin into conspicuous cables by clones 11 and 16, in contrast to cultures of M1 and M4 which showed largely diffuse fibronectin distribution. These observations raised 2 major aspects which we would like to address and discuss here. (*a*) To what extent are the observed differences in cell motility and adhesiveness *in vitro* related to the cell metastatic phenotype? (*b*) What may the primary difference be between the high- and low-metastatic variants which leads to the phenotypic alterations of cell adhesiveness, mode of interaction with the matrix, cytoskeletal organization, and motility? As far a the first question is concerned, it appears that several of the differences reported here between the cell variants of K-1735 melanoma may contribute, either alone or in conjunction with the cell metastatic capabilities. The reduced adhesiveness of cultured M1 and M4 cell lines together with their loose intercellular linkages and increased motility may be related to their increased capacity to migrate from the site of primary tumor, penetrate into the circulation, extravasate circulation, and actively move to their final site of growth. The validity of these correlations depends, of course, on the assumption that the behavior of these cells *in vivo* resembles their dynamic properties *in vitro*.

The contribution of fibronectin to the metastatic properties of K-1735 cells (if it has any) is not clear. It should be emphasized that both the high- and low-metastatic variants adhered and spread well on fibronectin carpet. However, whereas the interaction of the high-metastatic M1 and M4 cells with such carpet was relatively superficial, the low-metastatic clones 11 and 16 formed firm adhesion with the fibronectin carpet, followed by its displacement from contact regions under the cells. Do similar interactions occur when the different cells encounter fibronectin in connective tissue and basement membranes? It is expected that, under such conditions, the low-metastatic cells will attach firmly to the matrix and may be immobilized on it, while the high-metastatic counterparts may move freely on or through it. It should, however, be clearly pointed out that, as expected from a multistage process in which distinct steps may become rate limiting in different tumors, the relationships between metastatic potential and cellular architecture and dynamics may vary considerably from one tumor system to the other. Furthermore, a reduction in cellular adhesiveness, cytoskeletal rearrangement, and formation of fibronectin matrix were often associated in the past with the "transformed phenotype" as compared to the so-called normal state (5, 10, 19, 42, 46). On the basis of our results, we put forward the possibility that these interrelated properties are characteristic not to the transformed state *per se*, since the low- and high-metastatic cells are equally tumorigenic, but to dynamic properties of the tumorigenic cells, which is possibly directly related to their metastatic phenotype. This notion is in line with several other cell characteristics considered previously to be markers of transformation and is not directly involved in the basic lesion(s) in growth control of the neoplastic state (3, 4, 44).

The second aspect to be discussed here deals with the causal interrelationships between the various cellular properties of the low- and high-metastatic cells, namely, the interrelationships between a particular type of cell adhesion (focal contact formation), the organization of actin bundles, the active rearrangement of the extracellular matrix, and movement. The observations reported here are in agreement with the notion that rapidly moving cultured cells (both cell lines or primary cultures) have relatively poor stress fibers, while stationary cells often display large substrate contacts and many cytoplasmic actin bundles.

The adhesion pattern, cell mobility, and cytoarchitecture of the different K-1735 melanoma variants were not altered when the cells were plated on fibronectin-coated solid substrate or on matrix produced by the low-metastatic cells instead of the control glass or plastic, suggesting that the reported differences between high- and low-metastatic cells are not merely due to a response of the cells to an altered extracelluar matrix but are rather primary properties of the cells.

Differences in the display of fibronectin similar to those reported here for the high- and low-metastatic variants of K-1735 melanoma were described previously for "transformed" cells, as compared with the "nontransformed" counterparts (5, 8, 46). Furthermore, it has been reported that cell lines derived from nonmalignant tissues or primary carcinomas display larger amounts of fibronectin, as compared to the metastatic phenotype (21, 37); these correlations do not seem to be general; and examples were described in which no apparent relationships were noticed between the malignant potential of cells and the production or secretion of fibronectin (23, 26). Stenman and Vaheri (38) have reported that the relative display of fibronectin on benign and malignant cells depended primarily on the histogenic origin of the different cells. Our results support the latter notion that, while fibronectin distribution in the K-1735 melanoma is related to the metastatic properties, this correlation is not general, and in 2 other tumor systems tested (B16 melanoma and UV-2237 fibrosarcoma), both high- and low-metastatic variants display low levels of fibronectin (not shown).

In conclusion, we propose that the mode of cell adhesion [shown in our previous study (32)], rearrangement of extracellular matrix, assembly of actin into bundles, and locomotion are all closely interrelated cell properties. Among these, the capacity to develop tight substrate contacts (focal contacts) appears to have a significant primary effect on the subsequent formation of actin filament bundles or on the capability of the cells to reorganize the extracellular matrix (for further discussion, see Refs. 6 and 16). However, still unclear is the primary molecular basis leading to the altered adhesiveness of the various metastatic cell variants. Elucidation of such molecular events during contact formation may further our knowledge not only on the general mechanisms of cell attachment, but also on the related cellular dynamics including those involved in metastatic dissemination.

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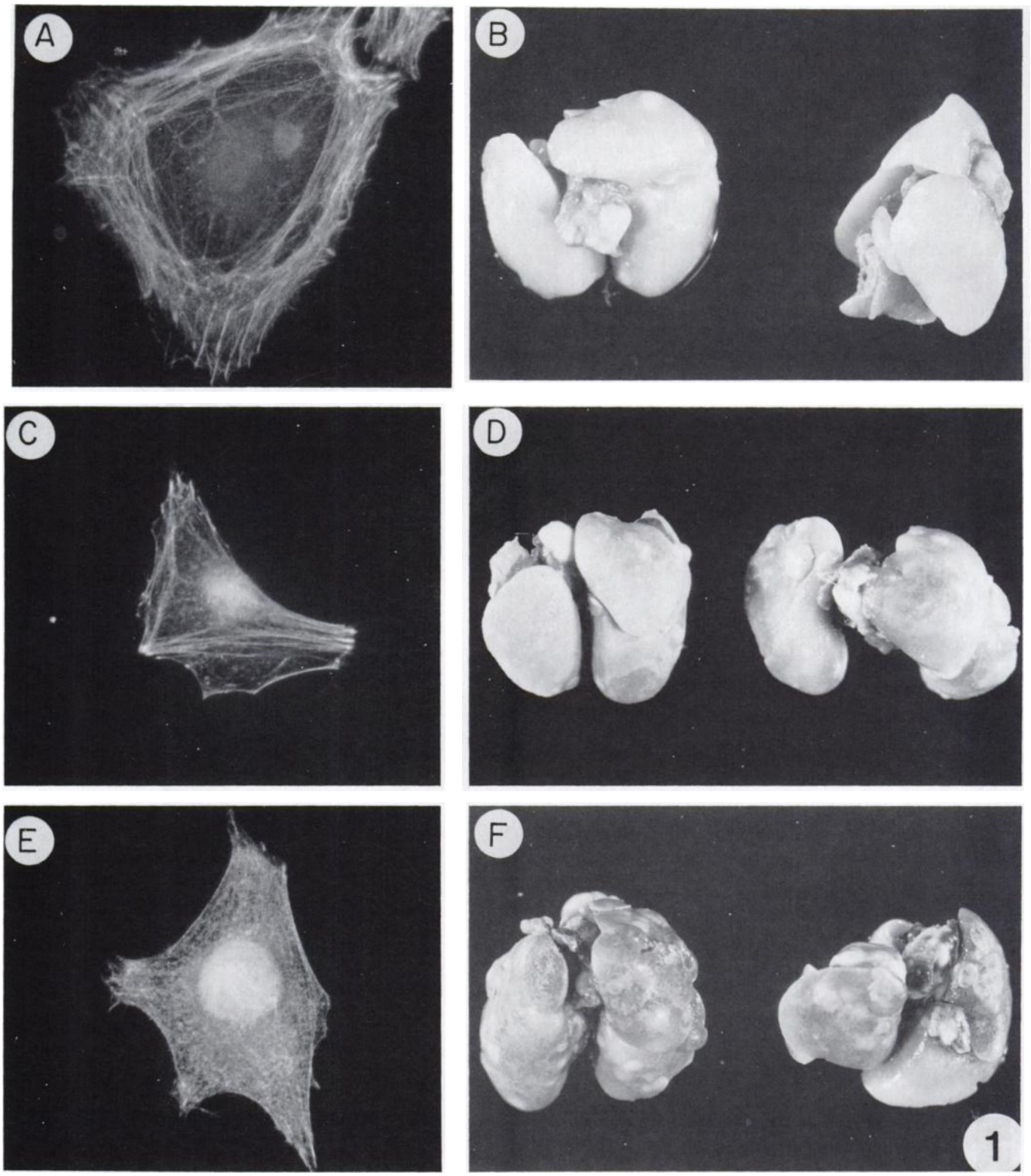


Fig. 1. Distribution of actin (*A*, *C*, and *E*) in 3 different clones of K-1735 melanoma and representation of the lung colonization capability of such clones (*B*, *D*, and *F*). The cells were permeabilized with detergent, fixed, and then labeled fluorescently with rhodamine phalloidin (1.5 μg/m). The lungs were excised from the mice 30 days after i.v. injection of 10^5 cells (0.2 ml PBS), washed, and fixed in Bouin's. *A*, *C*, and *E*, × 800; *B*, *D*, and *F*, × 2.

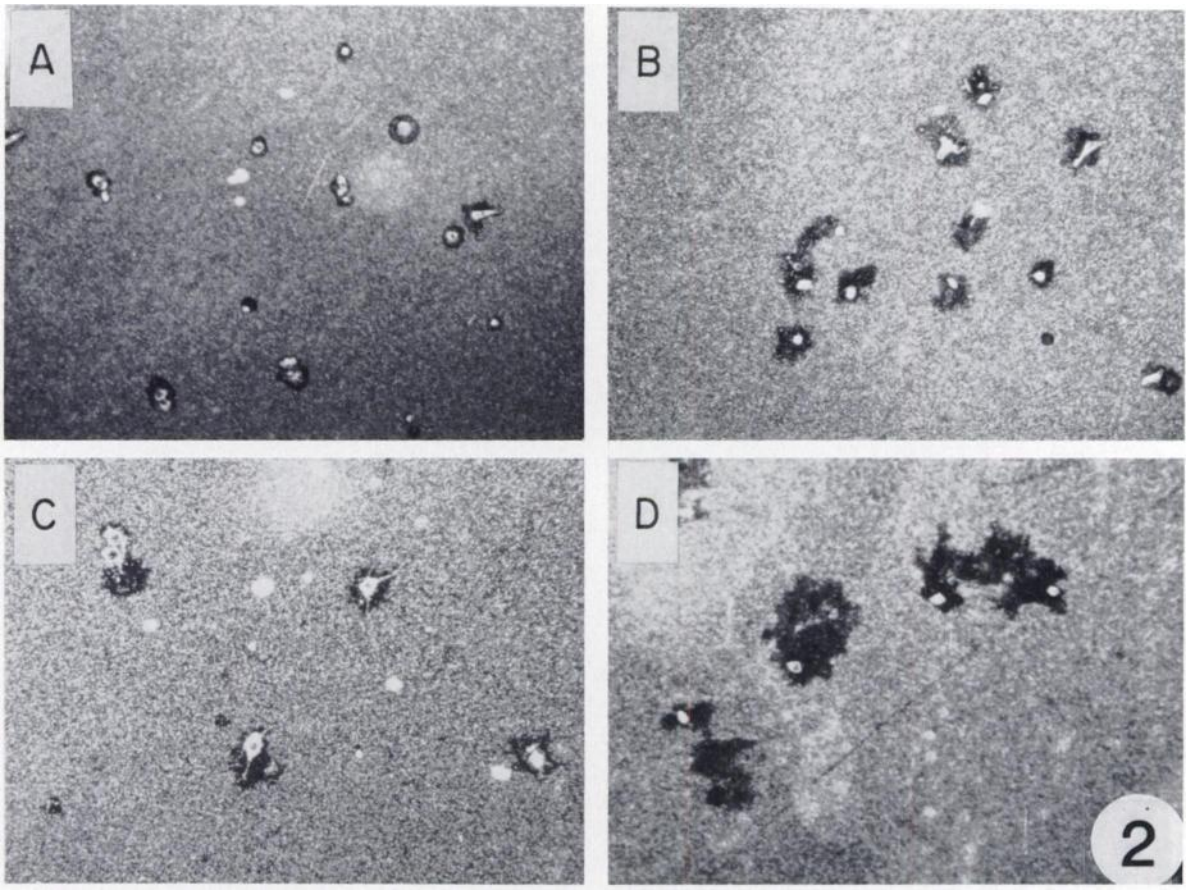


Fig. 2. Phagokinetic tracks formed by the low-metastatic clone 11 (*A* and *C*) and the high-metastatic line M1 (*B* and *D*) of K-1735 melanoma. The cells were fixed with formaldehyde, 24 hr (*A* and *B*) and 72 hr (*C* and *D*) after plating, and photographed under dark-field illumination. × 63.

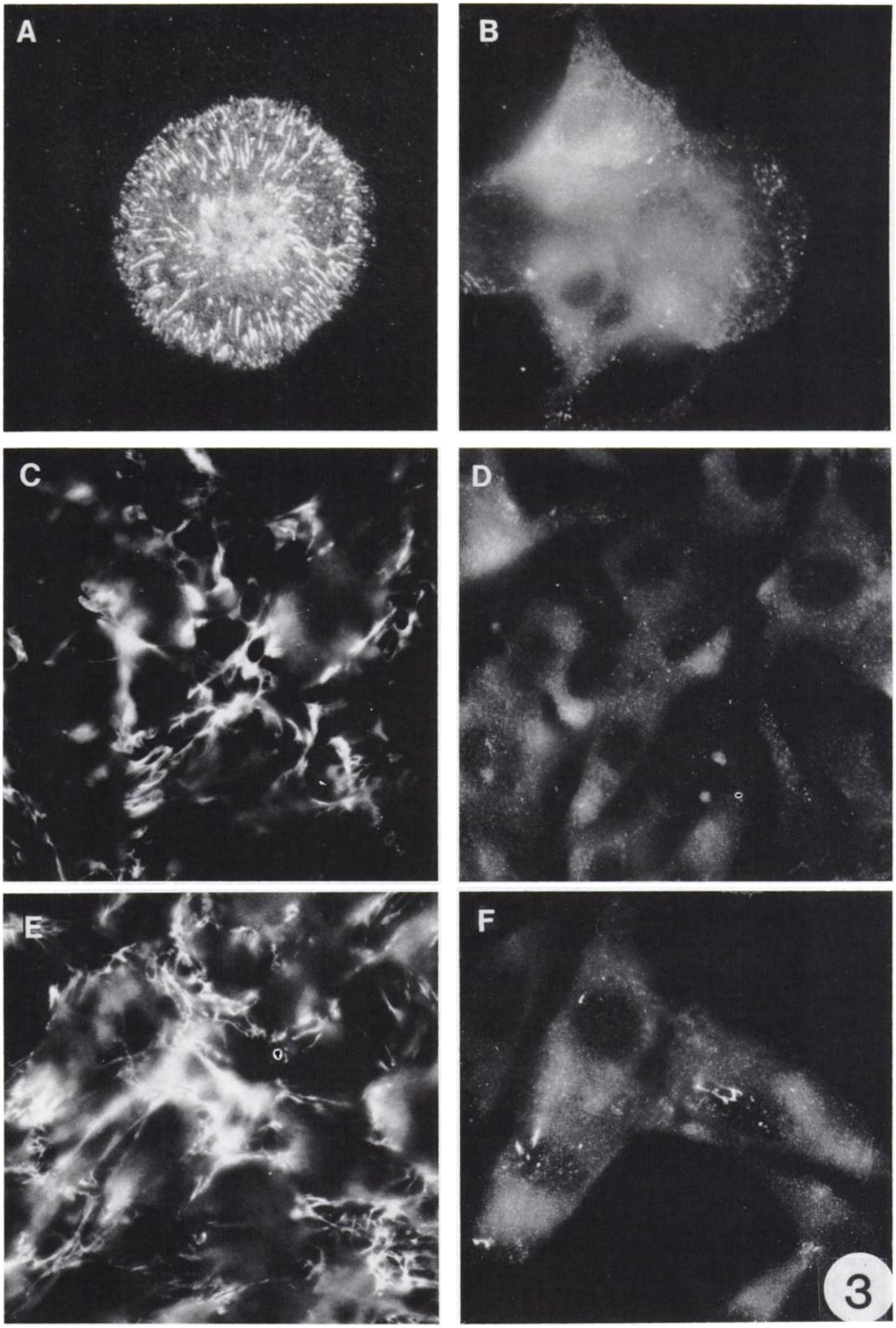


Fig. 3. The pattern of distribution of fibronectin in cultures of high-metastatic clones (*B*, *D*, and *F*) and low-metastatic clones (*A*, *C*, and *E*). Two high-metastatic clones were used: K-1735 M4 (*B* and *D*) and K-1735 M1 (*F*). The low-metastatic clones tested were K-1735 Cl-11 (*A* and *C*) and K-1735 Cl-16 (*E*). The cells were fixed and immunolabeled for fibronectin 1 day (*A* and *B*) or 5 days (*C* to *F*) after plating, using affinity-purified rabbit anti-human CIG antibodies and rhodamine-labeled goat anti-rabbit IgG. × 980.

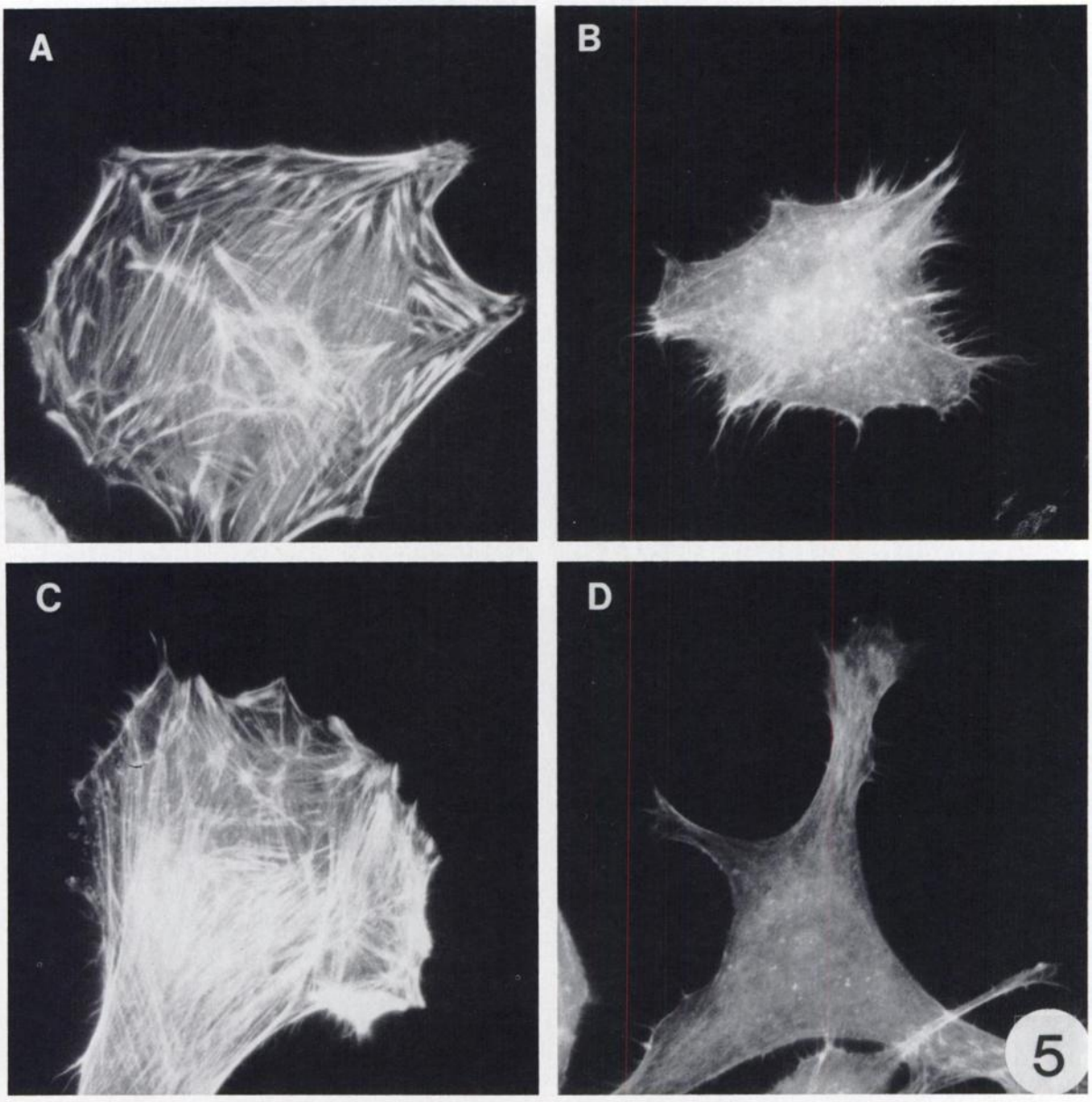


Fig. 5. The distribution of actin in low-metastatic cells of K-1735 Cl-11 (*A* and *C*) or the high-metastatic line M1 (*B* and *D*). The cells were seeded on regular glass coverslips (*C* and *D*) or on coverslips coated with fibronectin (15 μg/ml) (human CIG). After 24 hr, the cells were permeabilized, fixed, and labeled with rhodamine phalloidin. Notice that there was no significant effect to the fibronectin carpet on the organization of actin; in particular, the high-metastatic cells were not induced to form extensive arrays of actin bundles comparable to those of the low-metastatic cells. Identical results were obtained with K-1735 M1 and Cl-16. × 980.

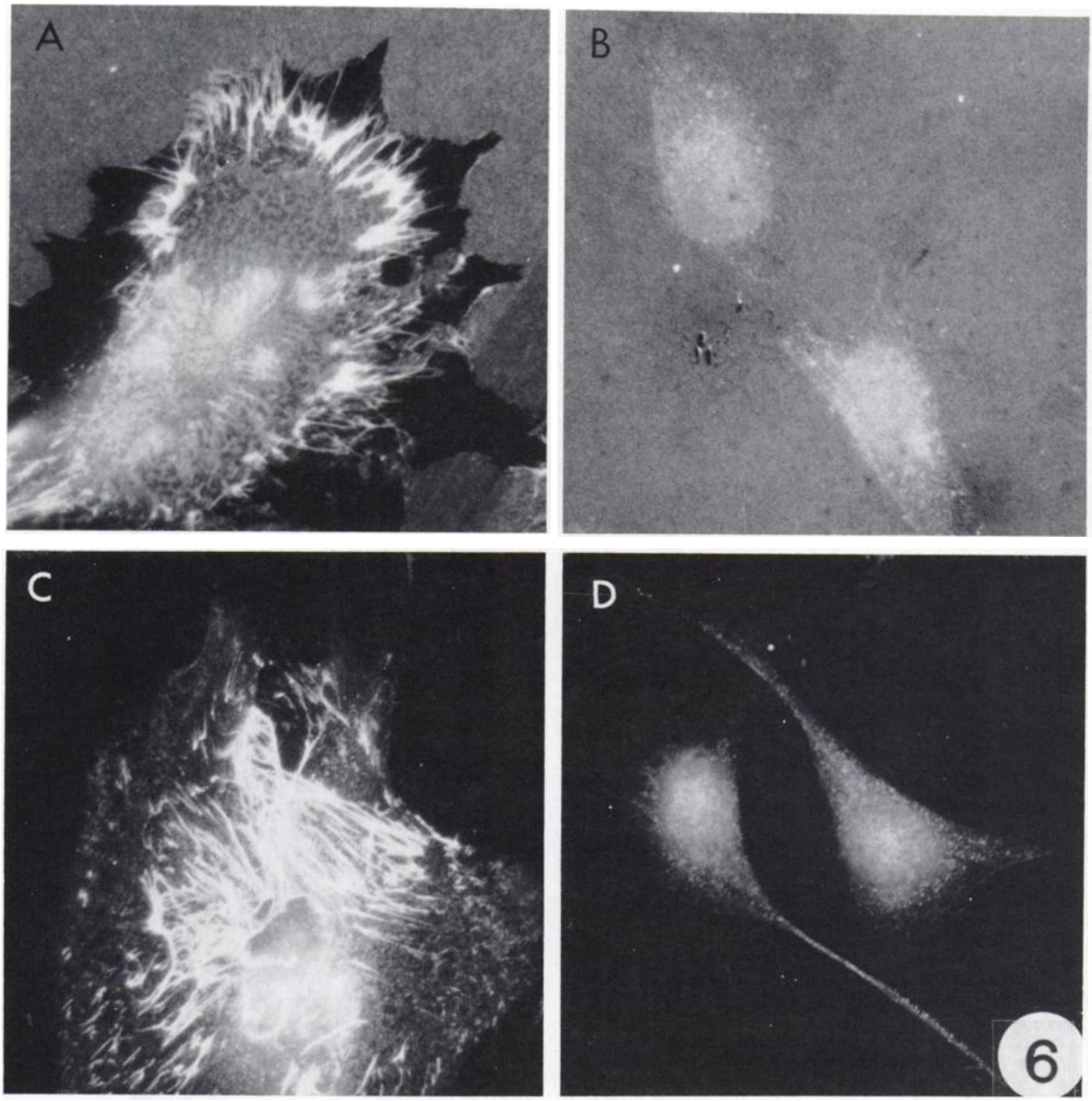
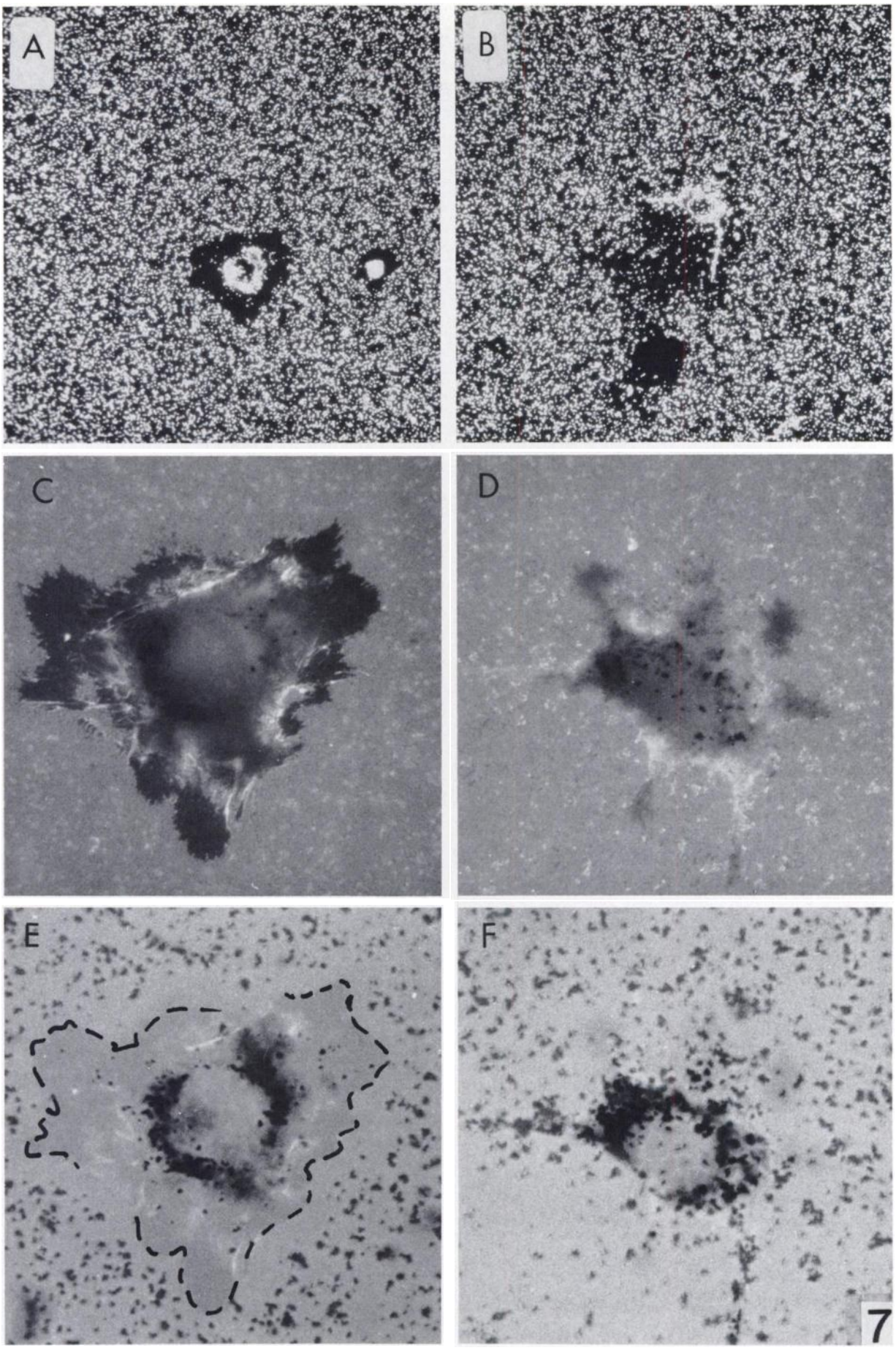


Fig. 6. Cells of the low-metastatic K-1735 Cl-11 (*A* and *C*) and high-metastatic K-1735 M1 (*B* and *D*) seeded on glass coverslips coated with human CIG (*A* and *B*) or uncoated (*C* and *D*). The cells were permeabilized with Triton X-100 24 hr after plating, fixed, and immunolabeled for fibronectin. The low-metastatic cells showed extensive formation of fibronectin cables concomitantly with removal of fibronectin from the underlying carpet. The high-metastatic cells did not form fibronectin cables nor did they significantly perturb the uniform layer of substrate-attached fibronectin. × 980.

Fig. 7. Low-metastatic cells (*A, C,* and *E*) or K-1735 Cl-11 and high-metastatic cells (*B, D,* and *F*) or K-1735 M1 were plated on gold particle-covered fibronectin-coated substrate and fixed 24 hr after plating. After fixation, the coverslips were immunolabeled for fibronectin as described. The same region was observed and photographed under low-power dark-field illumination (*A* and *B*, × 240), by high-power fluorescence microscopy (*C* and *D*, × 980). Notice that the low-metastatic variant removed fibronectin from its surroundings and reorganized it into cables, while the high-metastatic M1 hardly perturbed the fibronectin carpet. Both cells, however, removed gold particles upon spreading or movement. The K-1735 M1 cells showed higher locomotor activity than did the low-metastatic counterparts. *E* and *F* were taken under bright-field and fluorescence illumination simultaneously. Comparison of the area from which the gold particles were removed to the area from which fibronectin was removed is outlined by the *broken line* (*E*). The gold particles, seen as dark dots under the bright-field illumination, indicated that gold particles were removed from a larger area, probably independently of the underlying fibronectin layer (*F*).


A
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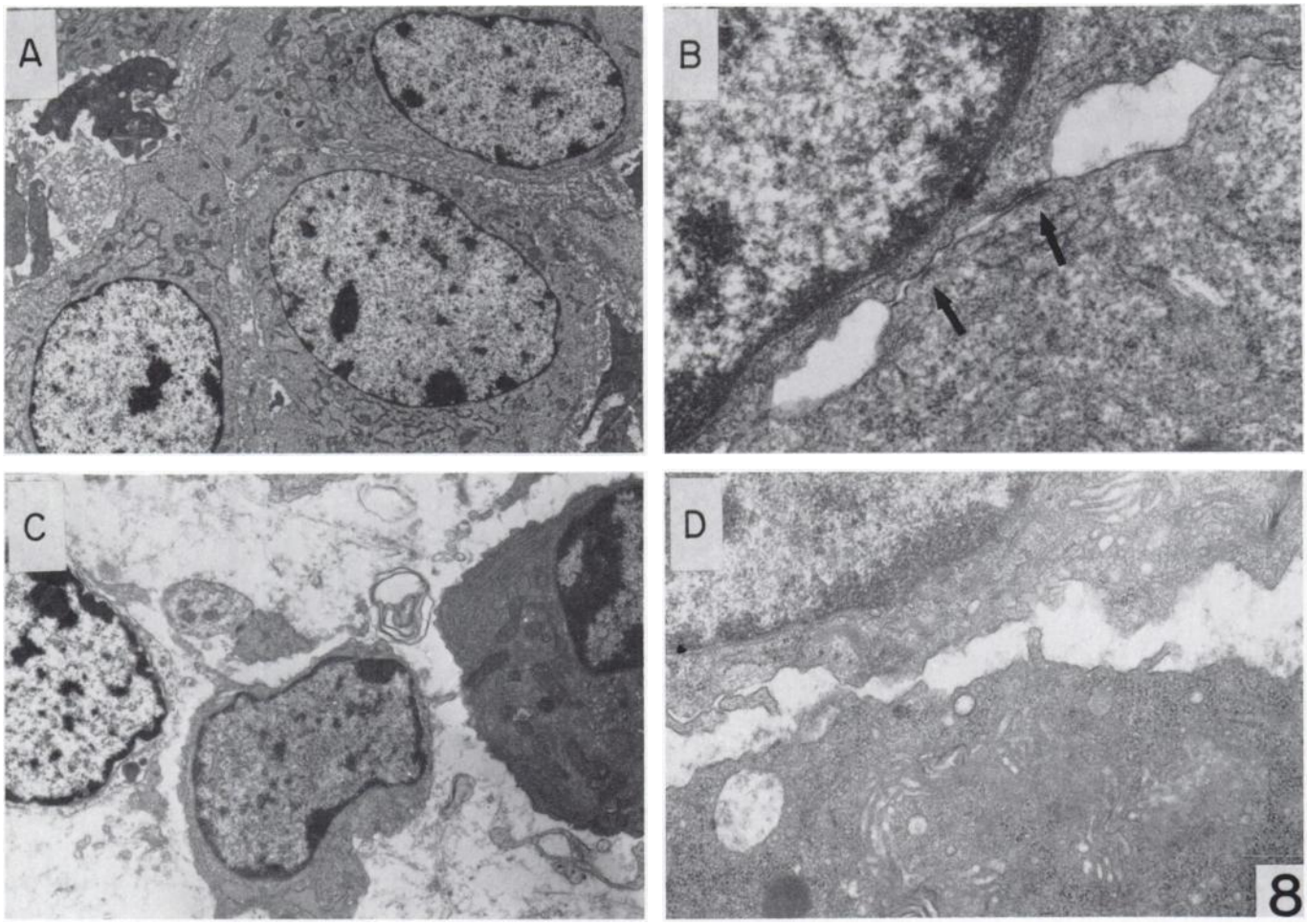

Fig. 8. Transmission electron microscopic examination of s.c. tumors produced by the low-metastatic Cl-11 of K-1735 melanoma (*A* and *B*) and the high-metastatic line M1 (*C* and *D*). The Cl-11 forms a more densely packed tumor with numerous focal intercellular contacts (*arrows*), while the high-metastatic M1 is characterized by disperse distribution of cells within the connective tissue and cellular debris with only few defined contacts. The cell distributions shown here were typical for these types of tumors, and similar patterns were obtained in different regions of the same tumor. *A*, × 3.0; *B*, × 3.1; *C*, × 7.8; and *D*, × 2.6 (× 10^3).